ISOLATION AND CHARACTERIZATION OF ALPHA-TERPINEOL DEHYDRATASE FROM *Pseudomonas gladioli*

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The enzyme-catalyzed hydration of the citrus by-product, limonene, to the important flavor and aroma chemical, α-terpineol, was investigated. Particulate-associated α-terpineol dehydratase was recovered from Pseudomonas gladioli, solubilized, and partially purified using detergent extraction and gel filtration chromatography.

Results of gel filtration chromatography suggested that α-terpineol dehydratase existed in two soluble forms in 1.0% (w/v) Triton X-100: a monomer and dimer with apparent molecular weights of 94,500 and 206,500 daltons, respectively. SDS-polyacrylamide gel electrophoresis revealed that a 92,000 dalton polypeptide was enriched during purification of the enzyme. Activity of α-terpineol

dehydratase was low in non-aqueous solvents. The reaction was not readily reversible, since enzyme-catalyzed dehydration of α-terpineol to limonene could not be demonstrated, even in media with reduced water concentration.

α-Terpineol dehydratase was characterized in buffers containing 0.1% (w/v) Triton X-100. In 10 mM MES, 10 mM BIS-TRIS PROPANE buffer, the pH optimum was 5.5 and the stability optimum was pH 8.0. The pI of the enzyme was between 6.5 and 6.8. The temperature optimum at pH 7.0 was 25°C in 10 mM HEPES buffer. Using temperature-activity data for 10-25°C, E_a and Q_{10} of α-terpineol dehydratase were determined to be 21.6 ± 2.9 $kJ \cdot mol^{-1}$ and 1.37 ± 0.07, respectively. Activity was inhibited by Triton X-100. The effects were an increase in apparent K_m and decrease in apparent V_{max}. Average apparent K_m of α-terpineol dehydratase was 2.18 ± 0.19 mM in 10 mM HEPES buffer, pH 7.0 containing 0.1% (w/v) Triton X-100.

α-Terpineol dehydratase stereospecifically catalyzed the hydration of (4R)-(+)-limonene to (4R)-(+)-α-terpineol or (4S)-(-)-limonene to (4S)-(-)-α-terpineol. The enzyme was also stereoselective, since the rate of hydration of (4R)-(+)-limonene was approximately ten times faster than the rate of hydration of (4S)-(-)-limonene.

Isolation and characterization of α-terpineol dehydratase has not been previously reported. Results of this study will increase the understanding of

microbiological hydrations of monoterpenes. Considerable economic potential exists, since the use of α-terpineol dehydratase to produce pure α-terpineol increases the value of limonene by 25%. This important natural process has an advantage over a chemical synthesis of being both stereo-selective and -specific.

INTRODUCTION

In recent years there has been increasing consumer preference for food products containing "natural" flavors over those containing artificial (synthetic) flavors. This preference has led to an increased demand for natural flavor and aroma chemicals (Buchel, 1989; Welsh et al., 1989). The increased demand for natural flavors has in turn placed more pressure on the production of flavor and aroma compounds by extraction processes from traditional raw materials such as plants. Use of these materials presents several problems. For instance, plants often contain low concentrations of the desired flavor compound, therefore making extraction expensive. Furthermore, the supply may be subject to seasonal, climatic, and geographical variation, as well as political and socio-economic stability of the producing regions (Armstrong and Yamazaki, 1986; Welsh et al., 1989). Such factors have caused an increase in the price of natural flavors and aromas.

According to the Code of Federal Regulations, compounds produced or modified by living cells or by their components, including enzymes, may be designated as natural (Code of Federal Regulations, 21 CFR 101.22.a.3.). In any case, the products can be considered natural if they are derived from

natural starting materials. Biotechnological or biological processes (processes involving the use of microorganisms, plant cell cultures, or enzymes) offer many possibilities for the production of natural flavor and aroma chemicals.

In addition to their use for production of flavor and aroma compounds, biological processes provide simple systems for studying the biosynthetic pathways involved in the formation of many important flavors and aromas. These processes have several advantages over alternate physical or chemical processes, the most important advantage being their ability to catalyze specific reactions, thereby avoiding potentially undesirable side reactions which may occur with less specific processing methods. Another advantage is that biological processes generally can be accomplished under mild conditions (i.e., ambient temperature, atmospheric pressure, and pH values near 7). This automatically results in lower energy consumption or decreased substrate and product damage.

The suitability of a process for production of flavor and aroma chemicals depends on the market demand (total usage), commercial value (price) of the chemical, and the technological state of the process. The use of a biological process for production of α-terpineol from limonene has economic potential because the annual consumption of α-terpineol is high, while at the same time its price is higher than limonene.

The monoterpene hydrocarbon limonene [1-methyl-4-(1-methylethenyl) cyclohexene, chemical formula $C_{10}H_{16}$], is ubiquitous in the plant kingdom. Limonene exists as both optically active and racemic mixtures (dipentene). Limonene is the main constituent of the terpene fraction of a number of essential oils, among which are citrus, caraway, dill, and American pine (Arctander, 1969a-c; Simonsen, 1947a). Citrus essential oils are unusual because they contain pure (4R)-(+)-limonene at concentrations approaching 95% for orange and grapefruit oils (Shaw, 1979). In addition to its high chemical and enantiomeric purity, limonene derived from citrus is in abundant supply, with 8.7 million kilograms being recovered during the 1988-89 Florida processing season (Anon, 1989). Limonene from citrus is also relatively inexpensive, the price being approximately 25% lower than the price of α-terpineol (Anon, 1990).

α-Terpineol [α,α,4-trimethyl-3-cyclohexene-1-methanol, chemical formula $C_{10}H_{18}O$] occurs in nature in both optically active and racemic forms; the (4R)-(+)-enantiomer has been identified as a component of petigrain, neroli, and sweet orange oils, the (4S)-(-)-enantiomer of camphor oils, and the racemic mixture of American pine and cajuput oils (Arctander, 1969d; Simonsen, 1947b). α-Terpineol is one of the most commonly used of all flavor and aroma chemicals. Its annual consumption for flavor purposes has been estimated at over 13,000 kg, which places it among the top 30 most commonly used flavors (Welsh et al., 1989).

α-Terpineol is commonly used in flavors such as lemon, lime, nutmeg, orange, ginger, peach, and spices (Arctander, 1969d).

Presently, α-terpineol is commercially available only as a racemic mixture, which is primarily recovered as a by-product from the pulp and paper industry. The properties of flavor and aroma compounds often depend on their optical purity. For example, (4S)-(+)-carvone has properties resembling caraway oil, whereas the properties of (4R)-(-)-carvone resemble spearmint oil. Biological processes are usually stereospecific; therefore it may be possible to produce pure (4R)-(+)-α-terpineol from (4R)-(+)-limonene by using this type of process. Cadwallader et al. (1989) demonstrated that Pseudomonas gladioli produces pure (4R)-(+)-α-terpineol from (4R)-(+)-limonene; however, the yield of α-terpineol was low due to the utilization of limonene by the bacterium for metabolic purposes. One way of directing the conversion of limonene toward the production of only α-terpineol would be by use of an isolated enzyme.

Suitability of an enzyme for the production of flavor and aroma chemicals depends on its physical and kinetic properties. It is necessary to isolate and purify enzymes to relate these properties to important parameters affecting reactions. For example, stability of enzymes should be relatively high under the process conditions. These conditions may include extremes of pH and temperature, as well as the presence of solvents or other common protein

denaturants. Enzymes should also be highly specific for the reaction of interest so that side products are minimized. When it is desirable to produce pure enantiomers from enantiomerically pure substrates, then the stereospecificity of the enzyme is important. Stereoselectivity is important when inexpensive racemic compounds are used as substrates instead of more expensive pure enantiomers.

The above discussion particularly relates to the importance of this research, which has the major objectives of (1) to isolate an enzyme from P. gladioli which catalyzes the hydration of limonene to α-terpineol, (2) to characterize some of its physical and kinetic properties, and (3) to define reaction conditions necessary for production of pure α-terpineol enantiomers.

LITERATURE REVIEW

Limonene

Physical Properties

Limonene (F.W. 136.24) is a colorless liquid with boiling point 178°C (CRC, 1986). It is practically insoluble in water (13 ppm at 25°C, Massaldi and King, 1973), soluble in alcohol, miscible with oils, but is poorly soluble in propylene glycol and glycerol (Arctander, 1969c).

Production

Limonene is produced in quantity as both racemic and (4R)-(+)-limonene. Racemic limonene (commonly referred to as dipentene) is isolated by fractional distillation of American Pine oil and rosin oils. It is also recovered as a by-product from the production of α-terpineol and from various synthetic products made from α-pinene or turpentine (Arctander, 1969a; Mattson, 1984).

(4R)-(+)-Limonene (commonly referred to as d-limonene) is recovered as a by-product from the manufacture of citrus molasses. Molasses refers to the concentrate produced from the press liquor, which is expelled from citrus waste residues after curing with small quantities of lime. Press liquor, which contains from 0.20 to 0.50% limonene, is normally passed through preheaters (115-140°C) for

pasteurization and scale removal and flashed at atmospheric pressure (100°C) to recover the limonene. The condensate (60-80% limonene) is put into a closed florentine-type tank which allows the limonene to float to the top of the tank where it is continuously decanted into storage tanks or drums (Kesterson and Braddock, 1976; FMC, 1976).

α-Terpineol

Physical Properties

α-Terpineol (F.W. 154.25) is a colorless, slightly viscous liquid with melting point 40-41°C and boiling point 220°C (CRC, 1986). It is slightly soluble in water (1980 ppm at 15-20°C, Seidell, 1928), but is soluble in alcohol, propylene glycol, and mineral oil (Arctander, 1969d).

Production

α-Terpineol is produced primarily in racemic form either by isolation from American Pine oil or by chemical synthesis from α-pinene via terpin hydrate (Arctander, 1969d) or directly to α-terpineol by hydration (Arctander, 1969d; Mattson; 1984). Non-commercial synthetic methods for the production of α-terpineol from limonene include (1) oxymercuration [aqueous tetrahydrofuran containing $Hg(OAc)_2$ and limonene] followed by reduction with $NaBH_4$ to give 70% α-terpineol (Brown et al., 1972) and (2) hydration of (4R)-(+)-limonene using chloroacetic acids to (4R)-(+)-α-terpineol (Matsubara et al., 1975). Methods involving acyclic starting materials include (1) cyclization of pentane tricarboxylic acid followed by esterification, via

the hydroxyester to the unsaturated ester, and then reaction with Grignard reagent to α-terpineol; and (2) by reaction of isoprene and methyl vinyl ketone with methyl magnesium iodide (Arctander, 1969d).

Biotransformation of Limonene

Biological approaches for the production of flavor and aroma compounds can be divided into biosynthetic and biotransformation methods. Biosynthesis refers to the production of chemical compounds by cell metabolism (fermentation or secondary metabolism by plant or microbial cells), whereas biotransformation is defined as the use of living cells or enzymes to perform specific modifications or conversions of chemical compounds (Welsh et al., 1989).

Research concerned with the microbial metabolism of terpenes was initiated by Bradshaw et al. (1959) and Seubert (1960). Since that time there has been a great number of publications in this area. Therefore, this section will be devoted to discussion of those studies involving the biotransformation of limonene.

Terpene flavor compounds are synthesized by a variety of higher plants and microorganisms. Low yield of terpenes produced by plant cell cultures or microbial fermentations, in addition to the abundance of natural terpenes from botanical sources, makes the development of biosynthetic processes for flavor and aroma terpene production unfavorable. Microorganisms and enzymes have the greatest potential for production of natural terpene flavors and

aromas via transformations of inexpensive natural terpene precursors to more valuable terpenes. Furthermore, microbes can achieve highly selective biotransformations of readily available substrates leading to products which are rare or otherwise difficult to synthesize.

Some bacteria and fungi are capable of utilizing terpenes as their sole source of carbon and energy. Terpene biotransformations are most often carried out by pseudomonads because these microbes have the ability to synthesize a wide variety of oxygenases and related enzymes necessary for the metabolism of many xenobiotics.

Bacteria and fungi differ in their metabolism of limonene. Bacteria appear to metabolize limonene primarily by progressive oxidation of the 7-methyl group. Generation of small amounts of neutral products occurs to some extent. The purpose of these "side" reactions is unknown, since these compounds are not further metabolized. Fungi attack limonene primarily by hydration of the double bond of the isopropenyl substituent or by epoxidation-hydrolysis of the 1,2-double bond. α-Terpineol and 1,2-diols are the most common products of fungal metabolism of limonene. Whether these compounds are intermediates or end products of minor pathways is uncertain.

Bacteria

The metabolism of limonene by a strain of Pseudomonas putida was studied by Dhavlikar and Bhattacharyya (1966) and Dhavlikar et al. (1966). The bacterium converted limonene

into several neutral and acidic products. The neutral products carveol, carvone, dihydrocarvone, 1-p-menthene-6,9-diol, cis- and trans-8-p-menthene-1,2-diol, and 8-p-menthen-1-ol-2-one were incapable of supporting the growth or respiration of the bacterium; however, the acidic products perillic acid, 2-hydroxy-8-p-menthen-7-oic acid, ß-isopropenyl pimelic acid, and 6,9-dihydroxy-1-p-menthen-7-oic acid were readily metabolized. These researchers proposed that P. putida metabolizes limonene by three distinct pathways, involving (1) allylic oxygenation, (2) oxygenation of the 1,2-double bond, and (3) progressive oxidation of the 7-methyl group to perillic acid which subsequently undergoes hydration, dehydrogenation, and hydrolysis. The reaction sequence of pathway 3 is illustrated in Figure 1.

The enzymes in pathway 3 were demonstrated in cell-free sonicates of the bacterium. The NADPH- and oxygen-dependent C-7 hydroxylation of limonene was shown to be associated with the 100,000 x g sediment of the sonicated cells. All other enzymes were present in the supernatant fraction. A cytochrome P-450 mixed function oxidase system was isolated from the same organism which catalyzes the C-7 hydroxylation of p-cymene (Madhyastha et al., 1968). Similarities between the structures of limonene and p-cymene suggest this cytochrome P-450 system, or one which is similar, catalyzes the C-7 hydroxylation of limonene to perillyl alcohol.

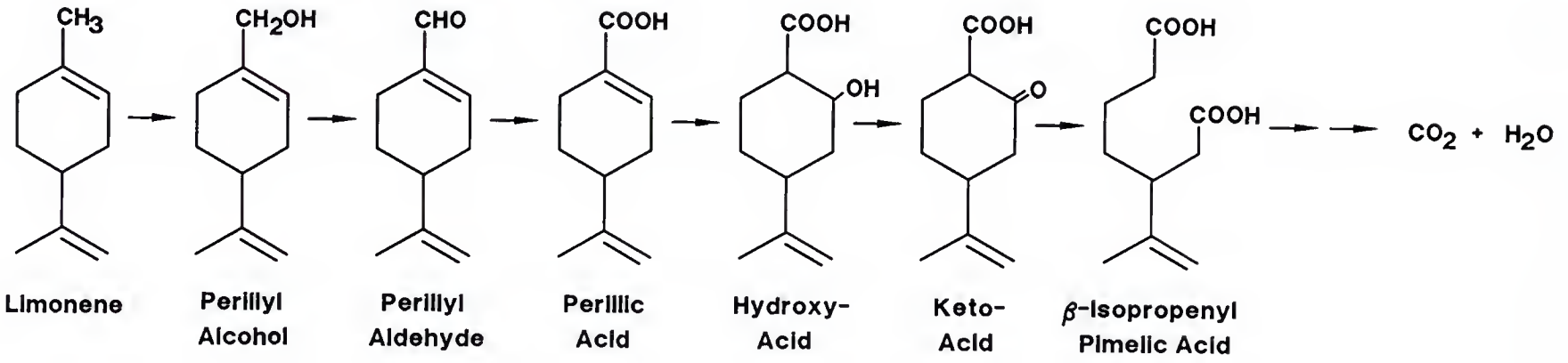


Figure 1. Reaction sequence of pathway 3 for the metabolism of limonene by Pseudomonas putida.

An NAD-linked alcohol dehydrogenase, perillyl alcohol dehydrogenase, was isolated from P. putida (Ballal et al., 1966). Partially purified (6-7 fold) enzyme was examined for its substrate specificity and cofactor requirement. The enzyme was absolutely specific towards NAD, with NADP being inactive. The enzyme demonstrated a broad substrate specificity. The following structural features of the substrate were found to favor dehydrogenation: (1) a primary alcohol group, preferably allylic to an endocyclic double bond, (2) a six-membered ring, and (3) an alkyl substituent in the para position.

An aldehyde dehydrogenase, perillyl aldehyde dehydrogenase, was also isolated from this organism (Ballal et al., 1967). The partially purified (10 fold) enzyme was highly specific towards NAD with relatively less activity with NADP. Besides perillyl aldehyde, this enzyme showed activity on several other aldehydes. No specific structural requirements were observed with respect to the substrate specificity of the aldehyde dehydrogenase as compared to that of the alcohol dehydrogenase.

The metabolism of limonene by Pseudomonas incognita was studied by Rama Devi and Bhattacharyya (1977a). Major conversion products were perillic acid and ß-isopropenyl pimelic acid. Small amounts of neutral products were recovered, but were not identified. Rama Devi and Bhattacharyya (1977b) demonstrated that the pathway of degradation of limonene by this bacterium occurred in the

following sequence: limonene → perillyl alcohol → perillic acid → ß-isopropenyl pimelic acid → → → CO_2 + H_2O, i.e. same as pathway 3 for the metabolism of limonene by P. putida (see Figure 1).

Fermentation of limonene by an enterobacterium was shown to result in the formation of dihydroperillic acid, perillic acid, and traces of unidentified neutral products (Dhere and Dhavlikar, 1970). The formation of perillic acid suggests that this organism metabolizes limonene using a similar pathway as P. putida.

Corynebacterium hydrocarboclastus has been reported to oxidize limonene to carvone (Takagi et al., 1969). The lack of proper controls (i.e., experiments to test for the autoxidation of limonene) and the very low yield of carvone (32 mg from 5.9 g limonene, in 30 hr) suggests that the appearance of carvone in the medium may have been due to chemical oxidation of limonene. Carvone has been shown to be an autoxidation product of limonene (Buckholz and Daun, 1978).

Cadwallader et al. (1989) demonstrated that Pseudomonas gladioli converts (4R)-(+)-limonene into (4R)-(+)-perillic acid, (4R)-(+)-α-terpineol, and at least one other unidentified acidic product. α-Terpineol was found to be resistant to further degradation, whereas perillic acid was readily degraded. This suggests that P. gladioli may metabolize limonene using a similar pathway as that described for P. putida.

P. gladioli is not the only bacterium capable of converting limonene into α-terpineol. Murdock et al. (1967, 1969) and Murdock and Hunter (1970) demonstrated that in some citrus oil emulsions limonene concentration diminished in direct proportion to an increase in α-terpineol concentration as a result of microbial growth. Several of these microbes were isolated but were not identified.

Fungi

Cladosporium spp. (T_{12}) has been shown to convert (4R)-(+)-limonene into pure (4R)-(+)-α-terpineol (Kraidman et al., 1969). Similarly, Penicillium digitatum (DSM 62840) was shown to produce (4R)-(+)-α-terpineol from either (4R)-(+)-limonene or racemic limonene (Abraham et al., 1985). This could be explained by the exclusive hydration of (4R)-(+)-limonene.

Epoxidation-hydrolysis of the 1,2-double bond of limonene leads to 1,2-diols (p-menth-8-ene-1,2-diols). Mukherjee et al. (1973) demonstrated that Cladosporium spp. (T_7) converts limonene to trans-1,2-diol plus a small amount of the corresponding cis-1,2-diol.

Corynespora cassiicola (DSM 62475) and Diplodia gossypina (ATCC 10936) were found to be stereospecific for the conversion of limonene to 1,2-diols. (4R)-(+)-limonene was converted to the (1S,2S)-diol, while (4S)-(-)-limonene yielded the (1R,2R)-diol (Abraham et al., 1984; Abraham et al., 1985).

The conversion of limonene by Penicillium italicum and P. digitatum resulted in low yields of 1,2-diols with respect to the other products isolated (Bowen, 1975). Cis- and trans-carveol were in highest yield followed by cis- and trans-p-mentha-2,8-diene-1-ol, carvone, p-menthena-2,8-diene-1-ol, carvone, p-mentha-1,8-diene-4-ol, and perillyl alcohol, respectively.

Plants

In addition to microorganisms, isolated plant enzymes and plant cell cultures have been shown to convert limonene to more important flavor compounds. Knorr et al. (1990) described the biotransformation of limonene to carvone by dill (Anethum graveolens) cultures. Maximum carvone production occurred with 5 mM limonene after 4 days of treatment. Prolonged treatment for 8 to 14 days resulted in degradation of carvone. It was proposed that this technique would be feasible for a continuous process involving continuous product recovery.

Microsomal preparations containing limonene hydroxylase activity were isolated from peppermint (Mentha piperita), spearmint (Mentha spicata), and perilla (Perilla frutescens) leaves (Karp et al. 1990). Each microsomal preparation generated only one product from limonene: trans-isopiperitenol from M. piperita, trans-carveol from M. spicata, and perillyl alcohol from P. frutescens. These compounds originated from hydroxylation of limonene at C-3, C-6, and C-7, respectively. The three hydroxylases had an

absolute requirement for molecular oxygen and NADPH. Both (4R)-(+)- and (4S)-(-)-limonene were hydroxylated by the three enzymes.

Enzymatic Production of Flavors and Aromas

Enzymes have traditionally been used as processing aids in the food industry. Most widely used enzymes are amylases, glucose oxidases, proteases, pectic enzymes, and lipases. Reviews on production and utilization of food enzymes are available (Wasserman, 1990; Whitaker, 1972). Largest use of enzymes for flavor production is for the production of syrups and sweeteners. Manufacture of glucose syrup and high fructose corn syrup is done solely by enzymatic means. This accounts for about 25% of the total usage of enzymes worldwide (West, 1987). Hydrolases, dehydrogenases and reductases have demonstrated the greatest potential for the production of flavor and aroma compounds. Excellent reviews of these enzymes are available (West 1987; Welsh et al., 1989).

Hydrolases

Hydrolases perform reactions such as esterifications, transesterifications, hydrolyses, and lactonizations (internal ester formation). Lipases, esterases, and proteases hydrolyze fat, carbohydrate, and protein molecules, respectively. Some of the most potent flavor compounds are esters. Natural esters retail at much higher prices than their synthetic counterparts. Lipases have been used to perform stereospecific hydrolyses and

esterifications to yield pure, optically active aliphatic and aromatic esters, alcohols, acids, and lactones (Welsh, 1989).

Lipases have been shown to mediate esterifications and transesterifications in organic media (Cambou and Klibanov 1984; Engel et al., 1989; Gerlach et al. 1988; Yokozeki et al., 1982). Enzymatic resolution of enantiomers can be achieved with some esterases and may represent an alternative to traditional chemical purification techniques (Engel et al., 1989; Omata et al., 1981)

Esterifications and transesterifications can be mediated by proteases. Proteases have been used to create unique hydrolyzed proteins which enhance or add flavors, particularly savory flavors, to foods. Use of proteases to produce modified food proteins has been recently reviewed (Alder-Nissen, 1985). Catalytic behavior of proteases can be modified by their use in organic solvents. Rate enhancements afforded by subtilisin and α-chymotrypsin for transesterification in octane were of the order of 100 billion-fold (Zaks and Klibanov, 1988). Similarly, peptide synthesis was favored instead of hydrolysis when α-chymotrypsin was reacted in ethanol or acetonitrile (Kisee et al., 1988).

Dehydrogenases and Reductases

Only two oxido-reductases have been seriously considered for flavor and aroma production (Welsh et al., 1989). These enzymes, alcohol dehydrogenase and alcohol

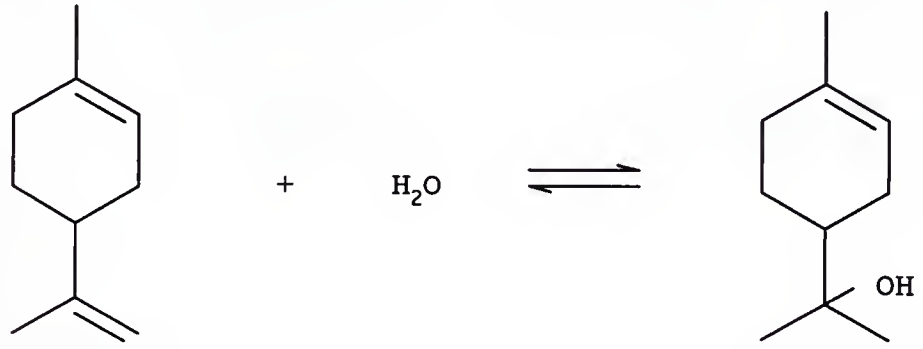
oxidase, perform the oxidation of aliphatic alcohols to their respective aldehydes. Alcohol dehydrogenase (ADH) can potentially be produced in commercial quantities from yeasts or plant cell cultures (West, 1987). The conversion of geraniol to geranial stands out as a typical conversion which can be mediated by ADH (Legoy et al., 1985).

The main drawback to using oxido-reductases is that these enzyme require cofactors for their catalytic function. Cofactors may act as either cosubstrates or active site participants. For many cofactors, especially those which are oxidized or reduced during the reaction, the initial cost of the cofactor prevents one-time usage on a large scale. High cost requires cofactor recycling if the process is to be economical. Recycling of NADH can be achieved by electrochemical means or substrate-driven reactions (Legoy et al., 1985; Nakamura et al., 1988); however, cofactor regeneration rates are not high enough for commercial purposes.

To expand the range of possible processes and to improve the economics of current enzyme processes, increased knowledge is needed concerning enzyme isolation and characterization, mechanisms of enzyme action, and incorporation of enzyme processes for natural flavor and aroma production. Specific needs are to understand the mechanisms of enzyme activation/inactivation, and to utilize enzymes in processes and redox reactions relevant to flavor production, including low-cost production and recycling of cofactors.

Enzyme-Catalyzed Eliminations and Additions of Water Kinetics

Aconitase, fumarase, enolase, and crotonase are examples of enzymes that catalyze reversible dehydration/hydration reactions. This type of reaction can be illustrated by the hydration of limonene to α-terpineol:



Limonene α-Terpineol

The biochemistry of enzymes is a subject of great depth and complexity. This section will attempt to describe only the aspects of enzyme kinetics applicable to the present study. For additional information, the reader may find greater detail elsewhere (Hammes, 1978; Segel, 1976; Walsh, 1979).

Kinetic equations can be readily derived assuming the simplest mechanism, i.e. that involving a single substrate and product. In fact, this mechanism is valid for the hydration of limonene to α-terpineol if it is assumed that water is always present in saturating amounts. The general mechanism is as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

$$-\frac{d[ES]}{dt} = (k_2 + k_{-1})[ES] - k_1[E][S] - k_{-2}[E][P] \qquad (1\text{-}1),$$

where S is the substrate, P is the product, E is the free enzyme, and ES is the enzyme-substrate complex. Since ES is rapidly destroyed, and therefore is present at very low concentration, the steady state condition d[ES]/dt = 0 can be assumed. Also, conservation of mass requires that

$$[E_t] = [E] + [ES] \qquad (1\text{-}2)$$

and

$$[S_t] = [S] + [P] + [ES] \qquad (1\text{-}3),$$

where E_t and S_t represent total enzyme and total substrate, respectively. Since [S] >> [ES] ≈ [E], [ES] in equation (1-3) can be neglected. The rate equation for the disappearance of S or the appearance of P can be written as follows:

$$-\frac{d}{dt}[S] = \frac{d}{dt}[P] = k_1[E][S] - k_{-1}[ES] \qquad (1\text{-}4).$$

Setting equation (1-1) equal to zero and substituting in equation (1-4) gives

$$-\frac{d}{dt}[S] = \frac{d}{dt}[P] = \frac{\{k_1k_2[S] - k_{-1}k_{-2}[P]\}[E_t]}{k_1[S] + k_{-2}[P] + k_{-1} + k_2} \qquad (1\text{-}5).$$

Measurements of the reaction velocity, v, are typically carried out at initial times, when [S] >> [P]. Therefore, the terms containing the concentration of product can be neglected in equation (1-5) and

$$v = -\frac{d}{dt}[S] = \frac{k_2[E_t]}{1 + (k_{-1} + k_2)/(k_1[S])} \qquad (1\text{-}6).$$

Defining $V_{max} = k_2[E_t]$ and $K_m = (k_{-1} + k_2)/k_1$, then equation (1-6) takes the form

$$v = \frac{V_{max}}{1 + K_m/[S]} = \frac{V_{max}[S]}{K_m + [S]} \qquad (1\text{-}7),$$

which is known as the Henri-Michaelis-Menten equation. Equation (1-7) shows dependence of v on [S], since as $[S] \rightarrow \infty$, $v \rightarrow V_{max}$, and also when $[S] = K_m$, $v = V_{max}/2$. Michaelis constant, K_m, is not an equilibrium constant but a "steady-state" constant and measures the ratio of steady state concentrations [E][S]/[ES]. Maximum initial velocity, V_{max}, is directly proportional to $[E_t]$.

The numerical value of K_m is important because it represents an approximate value for the intracellular concentration of the substrate. There would be little advantage in maintaining a substrate concentration higher than K_m since activity cannot exceed V_{max}. The difference between the activity at [S] equal to K_m and at [S] equal to $1000K_m$ is only two-fold. This property of K_m can be utilized when designing an enzymatic process. The K_m is constant for a given enzyme and can be used to make comparisons between enzymes from different sources. The K_m can also be used to indicate the relative suitability of alternate substrates for a particular enzyme. The substrate with the lowest K_m would have the highest apparent affinity for the enzyme. The "best" substrate is that which has the highest V_{max}/K_m ratio (Segel, 1976).

Nomenclature

Nomenclature and classification of enzymes is accomplished using rules and guidelines established by the Nomenclature Committee of the International Union of Biochemistry (1984). Enzymes which cleave C-C, C-O, C-N and other bonds by elimination leaving double bonds or rings, or conversely adding groups to double bonds, are classified as lyases. The systematic name is formed according to 'substrate group-lyase'.

Carbon oxygen lyases catalyze the breakage of C-O bonds leading to unsaturated products. In the case of hydro-lyases, this is by elimination of water or by elimination of an alcohol from a polysaccharide. The name 'dehydratase' is recommended for those enzymes which eliminate or add water to double bonds. The name 'synthase' may be used instead when it is desired to emphasize the synthetic aspect of the reaction or when the reverse reaction is much more important.

Where equilibrium warrants it, or where the enzyme has traditionally been named after a particular substrate, the reverse reaction may be used as the basis of the name using 'hydratase'. In the case of reversible reactions, the direction chosen for naming should be the same for all the enzymes in a given class, even when the direction has not been demonstrated.

Solubilization of Particulate-Associated Enzymes

This section is devoted to methods of approaching the problem of solubilizing enzymes or proteins which are associated with insoluble parts of the cell, such as membranes.

Particulate-associated proteins fall into two general categories. The first of these includes those proteins which, once solubilized, behave much like any other water-soluble enzyme. This category consists of two types of proteins: those which are present in a space surrounded by a membrane, but are not true components of the membrane itself; and those enzymes which are intrinsic components of a membrane, but upon being released are water-soluble. Procedures for the solubilization of these types of proteins have been summarized by Penefsky and Tzagoloff (1971).

The second category includes those proteins which are components of lipoprotein complexes. General procedures for the solubilization and purification of these proteins have been summarized by Tzagoloff and Penefsky (1971). Solubilization can be accomplished using detergents, in which the detergent binds to the protein and takes the place of the lipid-containing membrane, thus solubilizing the protein in the medium. During purification it is often necessary to include detergent in solutions to maintain protein solubility. Solubilization and purification of proteins using detergents is illustrated in Figure 2.

The use of chaotropic agents for the isolation of membrane-bound enzymes has been discussed by Hatefi and Hanstein (1974). Chaotropic agents decrease water structure thereby destabilizing membranes and enzyme complexes. Some commonly used chaotropic agents include sodium trichloroacetate, sodium and potassium perchlorate, and guanidine hydrochloride.

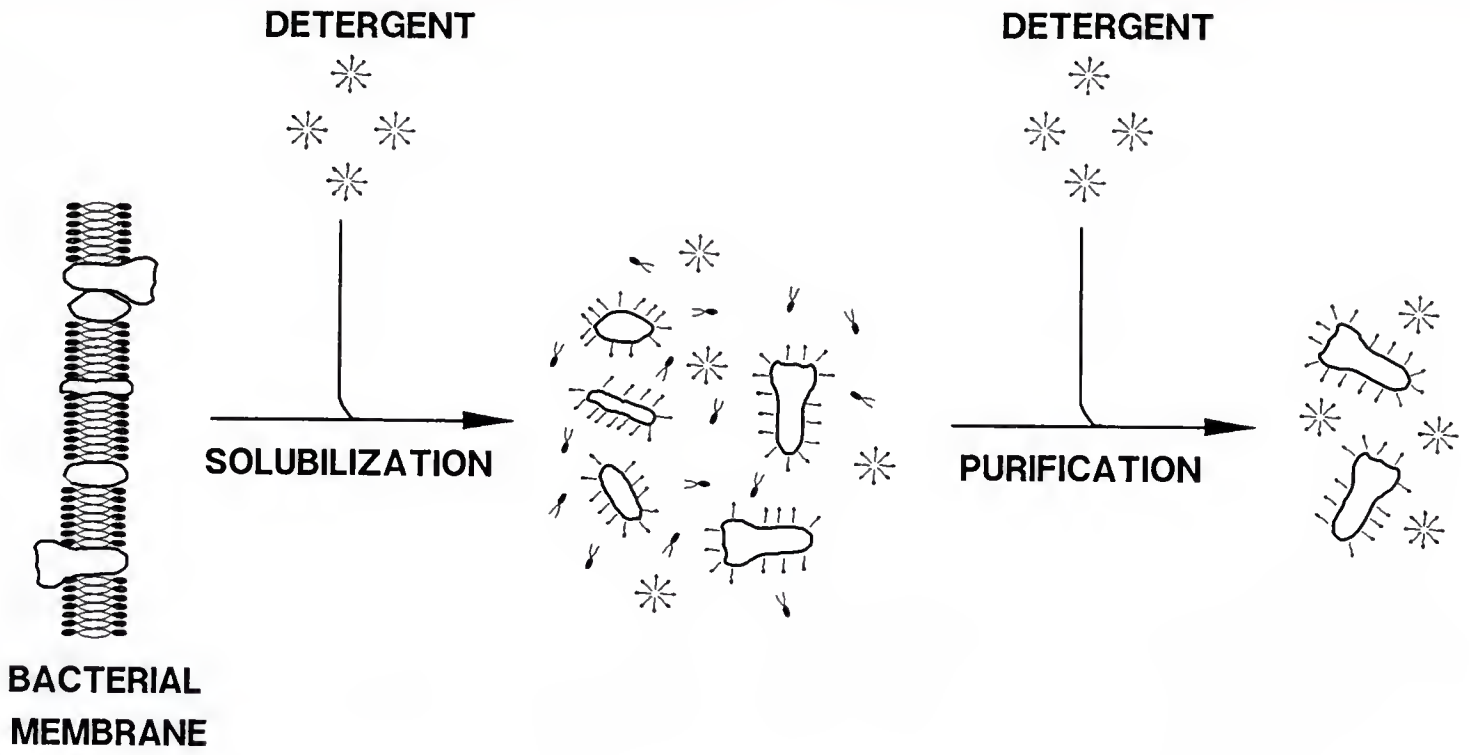


Figure 2. Solubilization and purification of particulate-associated enzymes using detergent solutions.

MATERIALS AND METHODS

Chemicals

(4R)-(+)-Limonene was a gift from Hercules Inc., Lakeland, FL; $[\alpha]_D^{24}$ +122.5 (neat), 99.0% pure by gas chromatography (GC). (4S)-(-)-Limonene was obtained from Aldrich Chemical Company Inc., Milwaukee, WI; $[\alpha]_D^{19}$ -86.6 (neat), 97.1% pure by GC. α-Terpineol (racemic) was a gift from International Flavors, Union Beach, NJ; $[\alpha]_D^{24}$ -10.2 (c=5.1, chloroform), 98.7% pure by GC.

(4R)-(+)-α-Terpineol was prepared by fermentation of (4R)-(+)-limonene by P. gladioli. Bacteria were grown in a group of 9 flasks as described under Growth and Collection of Bacteria, except that after an initial 24 hr incubation period 1 mL of (4R)-(+)-limonene was added to each flask and the flasks incubated for an additional 6 days. The culture broth was centrifuged for 20 min at 15,000 x g to remove bacteria. The bacterial pellet was resuspended in 200 mL distilled water and centrifuged as described above. The clarified culture broth and pellet wash supernatant were pooled and then extracted with diethyl ether using the method of Cadwallader et al. (1989). Crude α-terpineol extract (65% pure by GC) was purified by silica gel column chromatography. α-Terpineol in n-hexane (2.5 mL) was

applied to a slurry packed silica gel column (2.0 x 20 cm) which was equilibrated in n-hexane. The column was developed using the following sequence of solvents: 100 mL n-hexane, 100 mL of 50% n-hexane/50% methylene chloride, and 200 mL methylene chloride. Fractions (20 mL) were collected and analyzed by GC. Fractions containing the highest amounts of α-terpineol were pooled and the solvent evaporated under a stream of nitrogen to yield 0.2150 g of purified (4R)-(+)-α-terpineol; $[\alpha]_D^{24}$ +102.0° (c=1.1, chloroform), 94.0% pure by GC.

Microbiology

Liquid Mineral Medium Preparation

Liquid mineral medium (Monod and Wollman, 1947) was prepared by adding the following chemicals consecutively to 3 L of distilled water until completely dissolved: KH_2PO_4 (12 g), Na_2HPO_4 (108 g), $MgSO_4$ (10.8 g), NH_4Cl (16 g), $CaCl_2$ (8 mL of a 1% (w/v) solution), and $FeSO_4 \cdot 7H_2O$ (4 mL of a fresh 0.1% (w/v) solution). The solution pH was adjusted to 6.5 with 85% phosphoric acid and then diluted to 4 L to make 4X strength liquid mineral medium. To make single strength liquid mineral medium 1 L of 4X liquid mineral medium was diluted to 4 L with distilled water. The pH was measured and adjusted if necessary to pH 6.5 with 85% phosphoric acid.

Enumeration of Bacteria

Microbial populations (viable cells) were estimated according to procedures described by the American Public

Health Association (1989) using Standard Methods agar (BBL Microbiology Systems, Cockeysville, MD). Serial dilutions were made in 0.1% peptone. Aliquots of each dilution (0.1 mL or 1 mL) were transferred to duplicate petri dishes and pour plated with 15-20 mL of agar tempered at ca. 45°C. Plates were incubated at 30°C for 48 hr.

Bacterial growth was also monitored by measuring the absorbance (660 nm) of the culture broth.

Growth and Collection of Bacteria

P. gladioli was grown in 1 L Erlenmeyer flasks containing 250 mL liquid mineral medium, 0.5 mL sterile (4R)-(+)-limonene, and 25 mL inoculum. Flasks containing liquid mineral medium were sterilized at 121°C for 20 min. Limonene was filter-sterilized using a 0.22 μm MSI membrane filter (Micron Separations Inc., Westboro, MA). Flasks were incubated for 24 hr at 30 ± 1°C on an Environ orbital shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 200 rpm with 2 cm orbit. Inoculum was grown under the same conditions as described above. A total of 9 flasks were incubated per batch with one flask from each batch being used as the inoculum of the following batch.

Bacteria were collected by centrifuging the culture broth for 20 min at 15,000 x g in a Sorvall RC-5B refrigerated centrifuge (Dupont Co., Newtown, CT). The yield of bacterial cells from 8 flasks (2.2 L of broth) was about 6-7 g. Cells were stored at 4°C.

Analytical

Protein Determination

Protein concentrations were measured by the Coomassie blue protein assay (Bradford, 1976) using bovine serum albumin as the standard.

Enzyme Assays

In a typical enzyme assay the sample was transferred into a test tube (13 x 100 mm) and the final volume of the sample adjusted to 0.990 mL with 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid (HEPES) buffer, pH 7.0. The reaction was started by addition of 10 μL of substrate solution which contained 5 M limonene and 50 ppm decanol (internal standard) in absolute ethanol. The tube was then quickly sealed with a PTFE lined screw cap, mixed by vortexing, and placed in a temperature-controlled water bath. Unless otherwise stated, assays were done for 2 min at 25°C. The reaction was stopped by addition of 1 mL of n-hexane, which denatured the enzyme. Anhydrous sodium sulfate (3 g) was added to break the hexane emulsion. The hexane layer was transferred to a 1 mL vial and concentrated to about 0.1 mL under a stream of nitrogen. A small amount of anhydrous sodium sulfate was then added to the vial to completely dry the concentrated hexane phase before GC analysis.

Sample blanks were prepared by mixing 1 mL of n-hexane with the sample to denature the enzyme before adding 10 μL of substrate solution. Limonene blanks consisted of 0.990

mL buffer (typically 10 mM HEPES buffer, pH 7.0) and 10 μL of substrate stock solution. Limonene blanks were incubated under the same conditions as enzyme assays.

α-Terpineol concentration was determined by comparing the α-terpineol/decanol GC area ratio of the unknown with a calibration curve of α-terpineol/decanol GC area ratio versus α-terpineol concentration ($\mu mol \cdot L^{-1}$ or $nmol \cdot mL^{-1}$). A typical standard curve for α-terpineol is shown in Figure A-1. The curve is linear for concentrations of α-terpineol between 1.04 and 522 $\mu mol \cdot L^{-1}$.

The enzyme activity ($nmol \cdot min^{-1} \cdot mL^{-1}$) was calculated by dividing the concentration of α-terpineol ($nmol \cdot mL^{-1}$) by the assay time (min). Specific enzyme activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein) was calculated by dividing the enzyme activity ($nmol \cdot min^{-1} \cdot mL^{-1}$) by the protein concentration ($mg \cdot mL^{-1}$).

Gas Chromatography

The GC system consisted of an HP 5890 gas chromatograph equipped with a flame ionization detector (FID) and connected to an HP 3396A integrator (Hewlett-Packard Co., Avondale, PA). Chromatograms were analyzed using a computer integration program (Chrom-Perfect, Justice Innovations, Palo Alto, CA). Unless otherwise stated, a 0.53 mm i.d. x 15 m SE 54 fused silica capillary column (Alltech Associates, Inc., Deerfield, IL) was used for the separations. The film thickness of the liquid phase was 1.25 μm. Conditions were as follows: 5 μL injection with 1:3 split; helium carrier gas at 5.5 $mL \cdot min^{-1}$; injector port

at 175°C; detector at 250°C; column temperature programmed from 100°C to 200° at 10°C·min^{-1} with a final hold time of 5 min. A gas chromatogram of a typical enzyme assay extract is shown in Figure A-2.

Enzyme Purification

Buffer Preparation

All buffers were prepared at room temperature (20-25°C) using double distilled water. The pH of each buffer was adjusted with NaOH or HCl before its final dilution. The pH measurements were made at room temperature using an Accumet 915 pH meter (Fisher Scientific, Pittsburgh, PA) with automatic temperature compensation.

Isolation of Particulate-Associated Enzyme

The first step in isolation of a bacterial intracellular enzyme is to disrupt the organism. There are several techniques available for the disruption of bacteria: sonication, bead-milling, French press, and digestive enzymes such as lysozyme. Initial attempts to disrupt the bacterium involved a combined nonionic detergent-osmotic shock-lysozyme treatment as described by Schwinghamer (1980) and Scopes (1987). This technique was only partially successful in disrupting the bacterium and required a long incubation period. Ultrasonication was also attempted, but the conditions required to obtain adequate disruption of the bacterium were very vigorous and resulted in loss of enzyme activity, possibly due to heat denaturation. Small scale bead-milling resulted in adequate bacterial disruption and was used for initial studies.

The general procedure used for small scale bead-milling is described by Scopes (1987) and involves the use of small glass beads (0.1 mm diameter), which when vigorously stirred along with a suspension of bacteria disintegrate the bacteria by grinding action. Glass beads (8 g) were added to centrifuge tubes (28.5 x 104 mm) along with cell suspensions (1 g wet cells in 5 mL of 20 mM potassium phosphate buffer containing 5 mM $MgSO_4$ and 100 $\mu g \cdot mL^{-1}$ DNaseI) and the mixtures vortex-mixed for 10-20 min. Suspensions were centrifuged for 30 min at 20,000 x g in a Sorvall RC-5B refrigerated centrifuge in order to sediment the enzyme. All operations were carried out as close to 0°C as possible, except for centrifugation which was carried out at 4-6°C.

Large scale bacterial disruption was achieved using a commercially available bead-mill. Bacterial cells (20-30 g) were re-suspended in 200 mL of 50 mM HEPES buffer, pH 7.0 containing 0.2 mM dithioerythritol (DTE). The suspension was transferred, along with 500 g of dry glass beads (0.1 mm diameter), to a Bead-Beater (Biospec Products, Bartlesville, OK) equipped with a stainless steel chamber. The bacteria were disrupted for a total of 20 min (30 sec on, 60 sec off) with the chamber immersed in an ice bath. The cell homogenate was separated from the glass beads by vacuum filtration through nylon mesh filter cloth and the beads washed with an additional 50-100 mL of buffer. After adding 2.5 mL of 1 M $MgSO_4$ and 1 mL of a DNaseI solution (2500

units·mL^{-1} in distilled water) the cell homogenate was centrifuged for 20 min at 15,000 x g in a Sorvall RC-5B refrigerated centrifuge in order to sediment unbroken cells and large debris. The supernatant was further clarified by re-centrifuging under the same conditions. The purpose of DNaseI was to reduce the viscosity of the cell homogenate. Magnesium sulfate was added because Mg^{2+} is a required cofactor of DNaseI.

In order to sediment the membranous material containing the enzyme, the clarified supernatant was re-centrifuged for 2 hr at 25,000 rpm in a Beckman Model L5-65 ultracentrifuge using a Beckman SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA). The pellet from this centrifugation was re-suspended and washed twice with 70-75 ml of 10 mM HEPES buffer, pH 7.0 containing 0.2 mM DTE, with centrifugation the same as previously described. All operations were carried out as close to 0°C as possible, except for centrifugation which was carried out at 4-6°C.

Sucrose Gradient Centrifugation

The method of Osborn and Munson (1971) was used for sucrose gradient centrifugation with some modifications. Sucrose step gradients were prepared in ultracentrifuge tubes (14 x 89 mm) by layering 2.1 mL each of 50, 45, 40, 35, and 30% (w/v) sucrose over a cushion (0.5 mL) of 55% (w/v) sucrose. Sucrose solutions were prepared in 5 mM HEPES buffer, pH 7.0 containing 5 mM EDTA. Samples were prepared by re-suspending washed membrane pellets in 25%

(w/v) sucrose solution to a protein concentration of 2.2 mg/mL. One mL of sample was layered onto each of three tubes. One mL of 25% (w/v) sucrose was layered onto a forth tube for buoyant density determinations. Tubes were centrifuged for 18 hr at 35,000 rpm in a Beckman Model L5-65 ultracentrifuge using a Beckman SW 41 rotor (Beckman Instruments, Inc.).

Gradients were fractionated using an ISCO Model 640 density gradient fractionator (ISCO Inc., Lincoln, NB). Fractions (0.6 mL) were assayed for both enzyme activity and protein concentration. Buoyant densities were determined by measurements of refractive index using an Abbe Mark II refractometer (Rehert Scientific Instruments, Buffalo, NY). All operations were carried out as close to 0°C as possible, except for centrifugation which was carried out at 4-6°C.

Solubilization of Enzyme

Washed membrane pellets were re-suspended in 25-50 mL of 10 mM HEPES buffer, pH 7.0 containing 2.0% (w/v) Triton X-100, 0.5 M sodium trichloroacetate and 0.2 mM DTE. Suspensions were kept on ice and stirred for 30-60 min, then diluted with additional buffer until the protein concentration was equal to 5-10 $mg \cdot mL^{-1}$. Suspensions were centrifuged for 1 hr at 55,000 rpm (100,000 x g) in a Beckman TL-100 ultracentrifuge using a Beckman TLA100.3 rotor (Beckman Instruments, Inc.). All operations were carried out as close to 0°C as possible, except for centrifugation which was done at 4°C. Supernatants

containing the solubilized enzyme were decanted and kept at 4°C.

Gel Filtration Chromatography

Solubilized enzyme (20-30 mL) was applied to a 5.0 x 50 cm Spectra/Gel AcA 44 or AcA 22 column (Spectrum Medical Industries, Inc., Los Angeles, CA) which was equilibrated in 10 mM HEPES buffer, pH 7.0 containing 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02% (w/v) sodium azide. The enzyme was eluted from the column at a flow rate of 70-75 $mL \cdot hr^{-1}$ and 10 mL fractions were collected. Fractions were assayed for enzyme activity (30 min at 25°C) and protein concentration. All operations were carried out in a 4°C cold room.

Concentration of Gel Filtration Fractions

Gel filtration fractions containing enzyme activity were pooled and concentrated using a 150 mL capacity Omegacell ultrafiltration (UF) unit (Filtron Technology Corp., Clinton, MA) with a nominal molecular weight limit (NMWL) of 10,000 daltons. Nitrogen at 30-40 psi was used to pressurize the unit. Solutions containing the pooled fractions were concentrated to 10 mL and then 100 mL of 10 mM HEPES buffer, pH 7.0 was added and the solution reconcentrated to 10 mL. This procedure was repeated until the UF permeate had an absorbance (275 nm) of less than 1.0. All operations were carried out in a 4°C cold room.

Removal of Triton X-100 by Affinity Chromatography

Enzyme concentrate (10-15 mL) was applied to a 1.6 x 6.5 cm Spectra/Gel D column (Spectrum Medical Industries, Inc.) which was equilibrated in 10 mM HEPES buffer, pH 7.0. The enzyme was eluted from the column at a flow rate of 5 $mL \cdot hr^{-1}$. An additional 10 mL of 10 mM HEPES buffer, pH 7.0 was applied to the column in order to elute any remaining enzyme. The column was regenerated using the procedure recommended by the manufacturer, as follows: Column was washed with 1 bed volume distilled water, 1 bed volume of ethanol, 2 bed volumes of butanol, 1 bed volume of ethanol, 1 bed volume of distilled water followed by equilibration in buffer. All operations were carried out in a 4°C cold room.

Triton X-100 concentration in the Spectra/Gel D treated enzyme solution was estimated by measuring the absorbance (275 nm) of the UF permeate. Standard curves of absorbance versus Triton X-100 concentration for standard solutions and their respective UF permeates are shown in Figure A-3. The UF permeate curve was estimated by a straight line for Triton X-100 concentrations between 0 and 0.05% (w/v) and was used to estimate the Triton X-100 concentrations of sample enzyme solutions.

Enzyme Characterization

Molecular Weight Determination

The molecular weight (MW) of the native enzyme was determined by gel filtration chromatography. Solubilized enzyme (0.75 mL) was applied to a 1.0 x 50 cm Sepharose

CL-6B column (Aldrich Chemical Company, Inc.) which was equilibrated in 10 mM HEPES buffer, pH 7.0 containing 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02 % (w/v) sodium azide. The enzyme was eluted from the column at a flow rate of 5 $mL \cdot hr^{-1}$ and 0.4 mL fractions were collected. Fractions were assayed for both enzyme activity (30 min at 25°C) and protein concentration. The calibration curve was prepared with bovine thyroglobulin (MW = 669,000 daltons), horse spleen apoferritin (MW = 443,000), sweet potato ß-amylase (MW = 200,000), yeast alcohol dehydrogenase (MW = 150,000), and bovine erythrocyte carbonic anhydrase (MW = 29,000). Protein standards were obtained from Sigma Chemical Company, St. Louis, MO. The void volume was determined with Blue Dextran 2000. Protein standards (5 $mg \cdot mL^{-1}$) and Blue Dextran 2000 (10 $mg \cdot mL^{-1}$) were dissolved in gel filtration buffer. Blue Dextran 2000 fractions were monitored by measuring the absorbance (600 nm) after dilution of each fraction with 2.5 mL of distilled water. Gel filtration was carried out in a 4°C cold room.

The molecular weight of the enzyme in SDS denaturing conditions was determined by horizontal SDS polyacrylamide gel electrophoresis (SDS-PAGE) using ExcelGel SDS gradient (8-18% acrylamide) precast gels and buffer strips (Pharmacia LKB Biotechnology, Uppsala, Sweden). The electrophoresis system consisted of an LKB 2217 Multiphor II horizontal electrophoresis unit and an LKB 2297 Macrodrive S constant power supply (Pharmacia LKB Biotechnology). The running

conditions were as follows: 15°C; 50 mA constant current; 80 min. Gels were silver-stained using a PhastGel Silver Kit (Pharmacia LKB Biotechnology). The calibration curve was prepared using Bio-Rad SDS-PAGE high and low molecular weight standards (Bio-Rad Laboratories, Richmond, CA).

Isoelectric Focusing

The isoelectric point of the enzyme was determined by horizontal isoelectric focusing (IEF) using FisherBiotech agarose IEF gels and electrode solutions for pH range 3 to 10 (Fisher Scientific). The electrophoresis system was the same as that used for SDS-PAGE. Gels were equilibrated for 1 hr in a solution containing 2.5% (w/v) Servalyte 3-10 ampholytes (Fisher Scientific) and 1.0% (w/v) Triton X-100. In order to remove any salt or buffer ions which would disturb the pH gradient during focusing, enzyme solutions were dialyzed against either distilled water or 1.0% (w/v) glycine, pH 7.0 (Vesterberg, 1971). Enzyme concentrate (1 mL) was dialyzed against 2 x 250 mL of 1% (w/v) glycine buffer, pH 7.0 containing 1% (w/v) Triton X-100 (using dialysis tubing with MWCO 12,000-14,000 daltons) or against 2 x 250 mL distilled water containing 1.0% (w/v) Triton X-100 (Vesterberg, 1971). Dialyzed samples were filtered thru 0.2 μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI) to remove any debris before focusing. Samples were applied at various regions of the gel using a large template (1 x 1 cm wells), which was supplied with the IEF gels. Calibration curves were prepared using Bio-Rad broad range

IEF standards (Bio-Rad Laboratories). Gels were focused at 10°C using the following running conditions: 3 W/5 min, 5 W/30 min, 10 W/30 min, 15 W/30 min, and 25 W/5 min.

The gels were halved immediately after focusing. One gel half, which contained one lane of focused enzyme sample and two lanes of focused IEF standards, was soaked in a solution of 15% (w/v) trichloroacetic acid to fix the protein bands and then stained with Coomassie G-250. The other gel half, which contained 4 lanes of focused enzyme sample, was cut horizontally into 7.5 mm strips and each strip immediately suspended in 2 mL of 50 mM HEPES buffer, pH 7.0. The pH of each suspension was adjusted to 7.0 and the activity assayed (2 hr at 25°C).

pH Optimum and Stability

Enzyme concentrate (30 mL) was dialyzed against distilled water (1 L) containing 0.1% (w/v) Triton X-100 for 12 hr at 4°C using dialysis tubing with molecular weight cutoff (MWCO) of 12,000-14,000 daltons. The pH optimum of the enzyme was determined as follows: Aliquots of dialyzed enzyme (0.25 mL; 1.9 $mg \cdot mL^{-1}$ protein) were diluted to 2.5 mL in 10 mM 2-(4-morpholino)-ethane sulfonic acid (MES), 10 mM 1,2-bis[tris(hydroxymethyl)methylamino]-propane (BIS-TRIS PROPANE) buffer, with pH values ranging from 4.0 to 9.0 by increments of 0.5; The pH of each solution was measured and adjusted if necessary with 0.5 M HCl or 0.5 M NaOH to within 0.02 units of the indicated pH; Each solution was incubated for 30 min at 25°C and then assayed for activity (30 min at

25°C). The combined MES/BIS-TRIS PROPANE buffer was suitable for pH range 5.5 to 9.5.

The pH stability of the enzyme was determined using a similar procedure as above except for the following: Dialyzed enzyme aliquots (0.2 mL) were diluted to 2.0 mL in each buffer; solutions were incubated for 30 min at 25°C and then assayed for activity after adjusting the pH to 7.0 by addition of 0.5 mL of 1 M HEPES buffer, pH 7.0.

Temperature Optimum and Stability

Temperature optimum of the enzyme was determined by assaying the enzyme (1.7 $mg \cdot mL^{-1}$ protein in 10 mM HEPES buffer, pH 7.0 containing 0.1% (w/v) Triton X-100) for activity at various temperatures (10-50°C) after equilibration for 2 min. The temperature-activity data obtained from 10-25°C was used to estimate the activation energy (E_a) using an Arrhenius plot (log enzyme activity versus 1/T, where T is the absolute temperature in degree Kelvin). The slope of this plot is equal to $-E_a/(2.3R)$, where R is the gas constant (8.31441 $J \cdot K^{-1} \cdot mol^{-1}$). The activation energy ($J \cdot mol^{-1}$) was calculated from the slope.

The Q_{10} (the factor by which the rate increases by raising the temperature 10°C) of the enzyme was calculated using the following equation:

Q_{10} = [activity at (X + 10)°C]/[activity at X°C].

The temperature stability of the enzyme was determined as follows: Enzyme (2.0 $mg \cdot mL^{-1}$ protein in 10 mM HEPES buffer, pH 7.0) was incubated for 2 min at various

temperatures (20-60°C), immediately cooled on ice, and then assayed for activity at 20°C.

Stereospecificity and Stereoselectivity

Enzyme assays were the same as described earlier except that the substrate stock solution was composed of 0.02 M racemic limonene ($[\alpha]_D^{25}$ +0.7°; neat) and 1000 ppm (1S)-(-)-cis-pinane (internal standard) in absolute ethanol. Assays were done at 25°C for 5, 10, 20, and 40 hr. The GC system was the same as previously described except that a 0.254 mm i.d. x 30 m Cyclodex B fused silica capillary column (J & W Scientific, Inc., Folsom, CA) was used for the separation of limonene and α-terpineol enantiomers. The film thickness of the liquid phase was 0.25 μm. Conditions were as follows: 1 μL injection with 1:100 split; helium carrier gas at 1.26 $mL \cdot min^{-1}$; injector port at 200°C; detector at 250°C; column temperature programmed from 70°C to 200°C at $5°C \cdot min^{-1}$ with an initial hold time of 10 min and a final hold time of 5 min.

For determination of initial kinetic rates, enzyme assays were done using a substrate stock solution composed of 5 M racemic limonene and 500 ppm n-decanol (internal standard) in absolute ethanol. Assays were done at 25°C for 2, 5, and 10 min. GC conditions were the same as above except for the following: 1 μL injection with 1:10 split; helium carrier gas at 0.92 $mL \cdot min^{-1}$; column temperature isothermal at 115°C. A chiral gas chromatogram of a typical enzyme assay extract is shown in Figure A-4.

A calibration curve of α-terpineol/decanol GC area ratios versus α-terpineol concentration ($\mu mol \cdot L^{-1}$) is shown in Figure A-5. The actual concentration of each α-terpineol enantiomer was calculated from its relative concentration (i.e. the GC peak area of each enantiomer divided by the sum of the GC peak areas for both enantiomers). Enzyme activity was calculated using the same method previously described under Enzyme Assays.

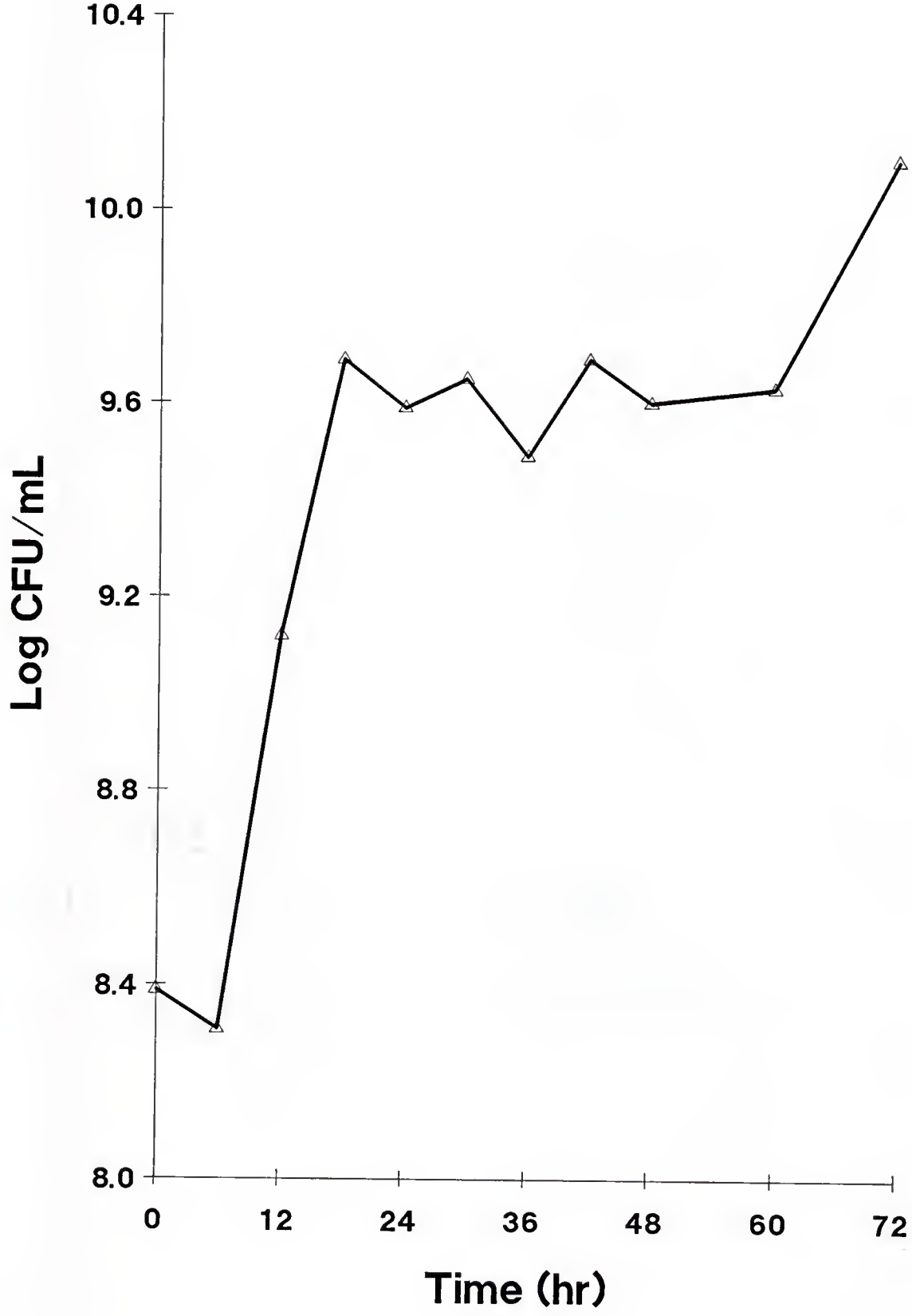
RESULTS AND DISCUSSION

Microbiology

Pseudomonas gladioli, which was used throughout this study, was the same bacterium described by Cadwallader et al. (1989). The bacterium exhibited optimum growth at pH 6.5 and 30°C in both tryptic soy broth and in liquid mineral medium containing 0.2% (v/v) limonene (Figure A-4). The concentration of limonene in the medium also affected the growth of the bacterium, with optimum growth occurring at a concentration less than 1.0% (v/v) (Cadwallader et al., 1989).

In this study the bacterium was grown exclusively in pH 6.5 liquid mineral medium containing 0.2% (v/v) limonene with incubation at 30°C. A typical growth curve of the bacterium under these conditions is shown in Figure 3. The exponential growth phase of the bacterium occurred between 6 and 18 hr with maximum number of viable cells occurring at about 24 hr. The ideal time for collecting cells is toward the end of the log phase before the growth rate slows, since this gives the highest yield of cells. The enzyme may not be at maximum concentration at this time. Preliminary studies determined which physiological state of the bacterium contained the most enzyme. Cultures of various

Figure 3. Growth curve of P. gladioli at 30°C in pH 6.5 liquid mineral medium containing 0.2% (v/v) limonene.


10.4
10.0
9.6
9.2
8.8
8.4
8.0
Log CFU/mL
0
12
24
36
48
60
72
Time (hr)

ages (24, 48, and 72 hr) were examined for cell density and enzyme activity. Enzyme assays (12 hr at 30°C) were conducted on cells suspended in 20 mM potassium phosphate buffer, pH 7.0 (1 g wet cells in 5 mL buffer). Results (Table 1) showed no significant difference ($\alpha = 0.05$) in enzyme activity between the 24 hr and 72 hr cultures; however, there was a significant decline ($\alpha = 0.05$) in cell density after 72 hr. It was concluded from these results that 24 hr was the most suitable time for collecting the bacteria.

Isolation of Particulate-Associated Enzyme

After centrifugation of the bacterial extract the majority of the enzyme activity was found in the particulate fraction. Initial small scale bead-mill experiments demonstrated that the enzyme activity of the particulate fraction was due to bound enzyme and not due to surviving bacteria. Enzyme activity and viable cells were determined before and after bead-milling for various times (Table 2). After 10 min of bead-milling greater than 99.999% of the bacteria were disrupted, while the amount of activity in the particulate fraction did not decrease. The supernatant fraction, which contained over 20 $mg \cdot mL^{-1}$ protein, showed only 7.9% of the total activity. The high protein concentration of the supernatant fraction was another indication of efficient cell disruption. Bead-milling the suspension for a total of 20 min resulted in an additional 99.98% reduction of viable cells over that observed at

TABLE 1

Cell density and enzyme activity of *P. gladioli* as a function of culture age

Culture Age (hr)	Cell Density ($mg \cdot mL^{-1}$)[a]	Enzyme Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ wet cells)[b]
24	3.44 ± 0.16	0.68 ± 0.06
48	3.53 ± 0.09	0.52 ± 0.06
72	2.79 ± 0.29	0.65 ± 0.07

[a]average ± standard deviation (n = 2)

[b]average ± standard deviation (n = 4)

TABLE 2

Amount of enzyme activity in particulate fraction as a function of extent of cell disruption

Extraction Time (min)	Enzyme Activity ($nmol \cdot min^{-1} \cdot mL^{-1}$)[a]	Viable Cells (Log $CFU \cdot mL^{-1}$)[b]
0	4.54 ± 0.17	13.2
10	4.7 ± 0.6	8.0
20	2.66 ± 0.08	4.2

[a]average ± standard deviation (n = 2)

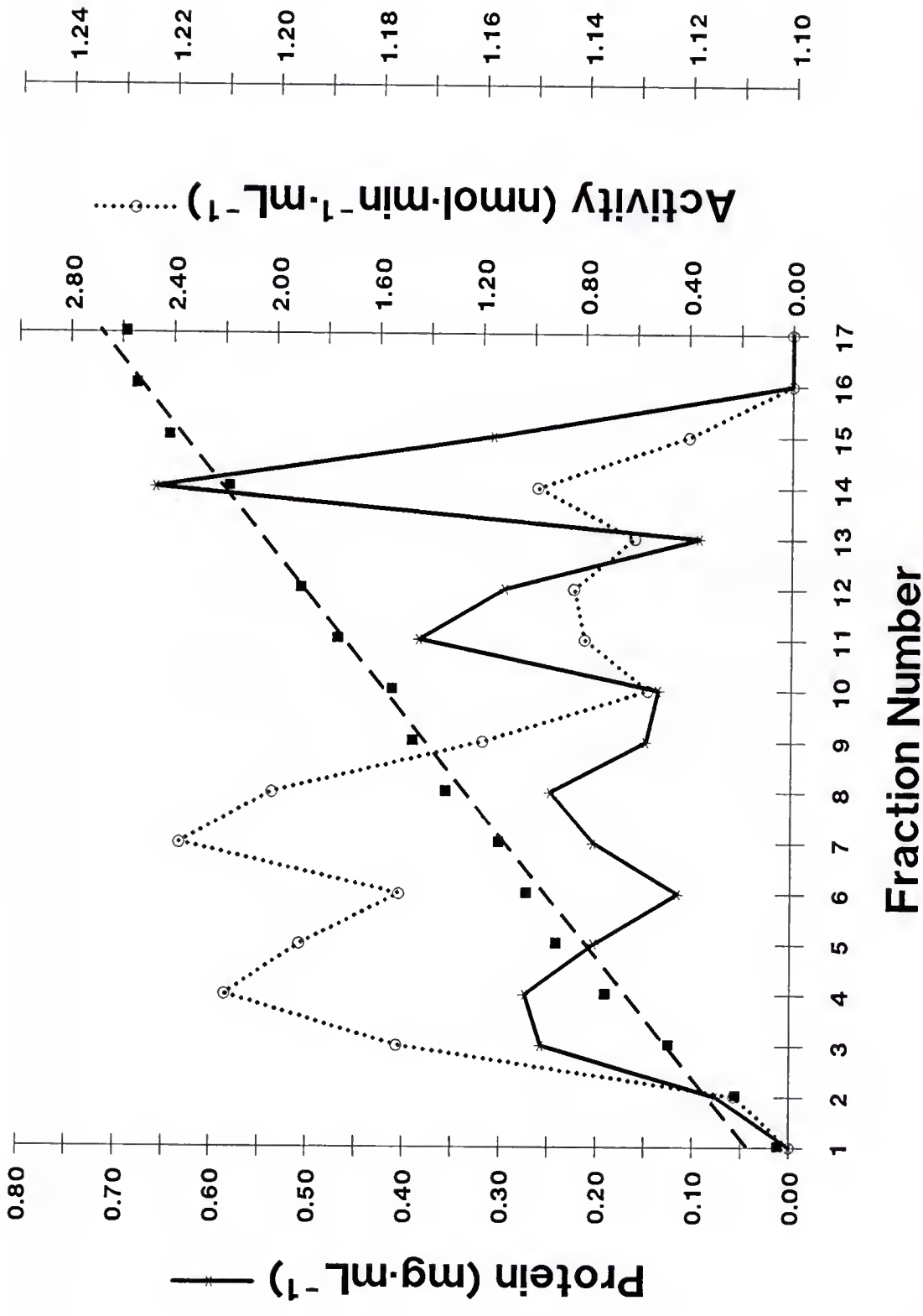
[b]average (n = 4)

10 min. The decrease in enzyme activity after 20 min was possibly due to denaturation of the enzyme, since only 2.6% of the total activity was found in the supernatant fraction.

Small scale bead-milling was very efficient, but the sample processing capacity was low. The technique was scaled up by utilizing a laboratory scale bead-mill. This unit was capable of disrupting up to 100 g of cells (wet wt.) in 200 mL medium. Disruption of 100 g of cells was not feasible because of the low yield of cells (6-7 g) from each batch of culture. The technique effectively disrupted 20-30 g of cells, but was apparently much more vigorous than the small scale method, since a higher centrifugal force was required to sediment the enzyme. This was not necessarily a disadvantage since a lower centrifugal force could be used to remove unbroken cells and large debris from the cell homogenate prior to sedimentation of the enzyme.

Isolated particulate-associated enzyme was partially purified by washing the pellet with a buffer which did not solubilize the enzyme. The particulate material was characterized by differential centrifugation. This technique was also evaluated for its potential as an enzyme purification step. Results of sucrose gradient centrifugation are shown in Figure 4. Protein was effectively fractionated into four bands. The intensity of these bands was highest at the sucrose layer interfaces. Enzyme activity was found in each band, with the majority of the activity being found in the two bands of lowest density.

Figure 4. Sucrose gradient centrifugation of particulate-associated enzyme.


Protein (mg·mL⁻¹)
Activity (nmol·min⁻¹·mL⁻¹)
Density (g·cm⁻³)
Fraction Number
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.00
0.40
0.80
1.20
1.60
2.00
2.40
2.80
1.10
1.12
1.14
1.16
1.18
1.20
1.22
1.24
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

The results of this experiment suggest that the enzyme was associated with particulate material of varying density and size. One explanation that might account for these results is that the enzyme was associated with a specific membrane of the bacterium and during bead-milling the membrane was sheared into smaller heterogeneous fragments. On the other hand, the enzyme may not have been membrane-bound, but instead became associated with its nearest hydrophobic neighbor after being released into the hydrophilic medium (buffer). The utility of sucrose gradient centrifugation as a purification step was limited by the low sample processing capacity and the time required to achieve equilibrium (18 hr). In addition, the degree of purification obtained was low (less than two-fold); therefore it was decided to solubilize the enzyme without any prior fractionation.

Enzyme Solubilization

Initial attempts to solubilize the enzyme involved those techniques described by Penefsky and Tzagoloff (1971) for water-soluble membrane proteins, but it was evident after several experiments that the enzyme was not characteristic of this type of protein. Extraction of the particulate-associated enzyme with concentrated salt (2 M NaCl and 6 M urea), alkali/EDTA, enzymes (phospholipase A_2, lysozyme, and pectolyase) and organic solvents (DMSO, ethanol, acetone, n-butanol, and dioxane) had no effect on the solubility of the enzyme. Extraction with organic solvents actually denatured the enzyme.

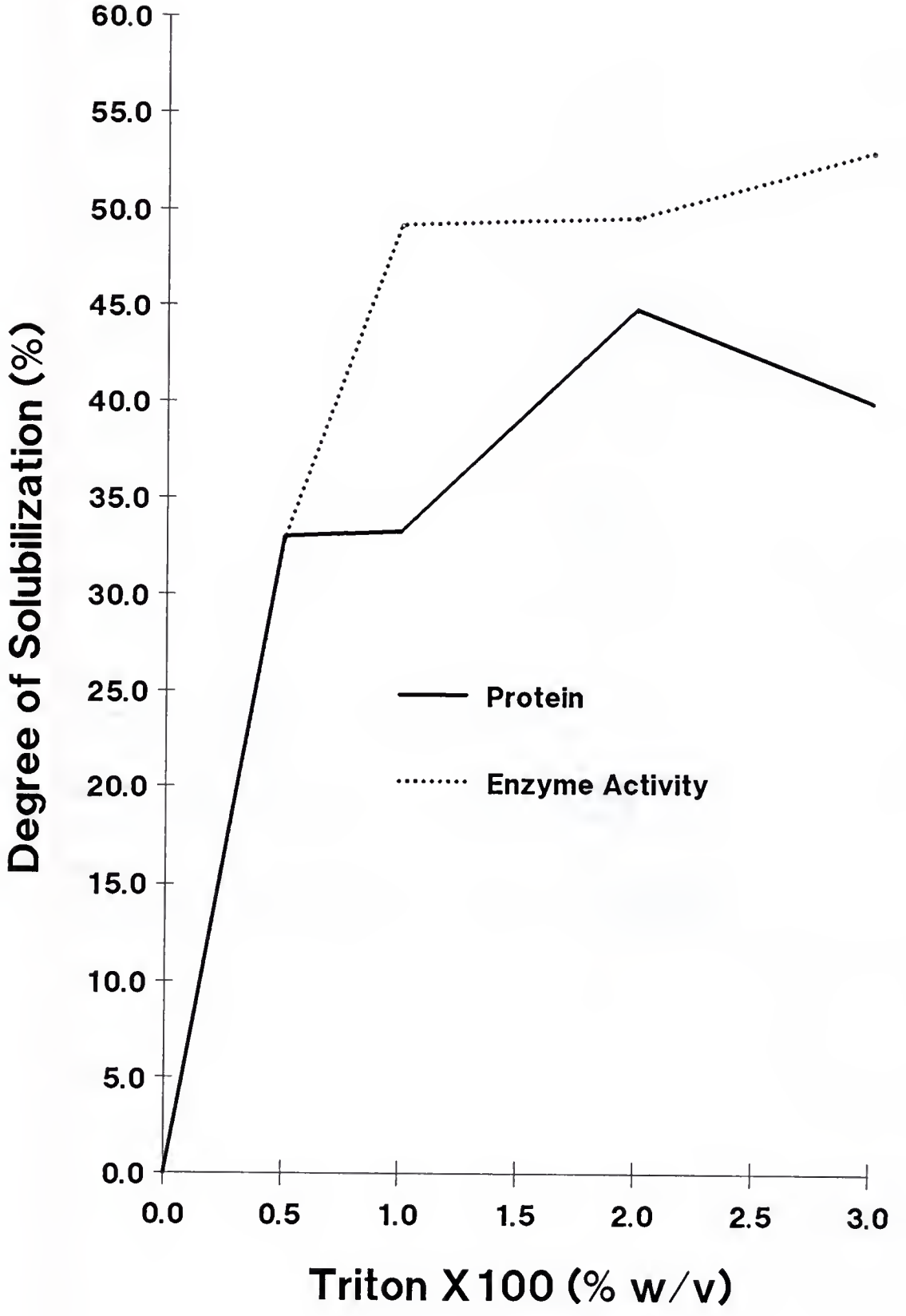
Partial solubilization of the enzyme could be achieved using detergents. Nonionic detergents, such as Triton X-100, Nonidet P-40, and n-octyl α-D-glucopyranoside were more effective then the ionic detergents; sodium cholate, SDS, and 3-[(3-choramidopropyl)dimethyammonio]-1-propanesulfonate (CHAPS). Triton X-100 was chosen for further enzyme solubilization studies. The degree of solubilization of the enzyme was found to be dependent on the concentration of Triton X-100 with 2.0% (w/v) giving more consistent results than 1.0% (w/v) (Figure 5). Nonionic detergents have been reported to be more effective when used in combination with salts (Tzagoloff and Penefsky, 1971); however, inclusion of various concentrations of NaCl, KCl or urea with 2% (w/v) Triton X-100 did not increase the solubility of the enzyme. Inclusion of the chaotropic agent, sodium trichloroacetate, in extraction buffers greatly increased the effectiveness of Triton X-100 for solubilizing the enzyme. Effect of including various concentrations of sodium trichloroacetate in solutions of 2.0% (w/v) Triton X-100 on the solubility of the enzyme is shown in Figure 6. Optimum concentration of sodium trichloroacetate was found to be 0.5 M when the protein concentration of the initial enzyme suspension was less than 10 $mg \cdot mL^{-1}$. Sodium trichloroacetate (0.5 M) alone had no effect on the solubility of the enzyme.

Gel Filtration Chromatography

Initial attempts to purify the enzyme using anion exchange (DEAE Spectra/Gel) and hydroxylapatite (Bio-Gel HTP) chromatography were unsuccessful due to irreversible adsorption of the enzyme, which occurred even when 1.0% (w/v) Triton X-100 was included in the elution buffers. The degree of success of gel filtration chromatography for enzyme purification depended on the concentration of Triton X-100 in the elution buffer. Gel filtration profiles of solubilized enzyme using Spectra/Gel AcA 44 with 0.5 and 1.0% (w/v) Triton X-100 in the elution buffer are shown in Figures 7a and 7b. With 0.5% detergent only 12-46% of the enzyme activity was recovered, whereas with 1.0% detergent yields close to 100% were obtained.

Low recovery of activity with 0.5% detergent was apparently due to adsorption of the enzyme to the gel. This is evident from the greater volume required to elute the enzyme with 0.5% detergent compared to with 1.0% detergent. With 0.5% detergent the enzyme eluted at the leading edge of the last and largest peak. This was the inclusion volume of the column and was composed mainly of excess detergent originating from the solubilized enzyme solution. After being adsorbed to the gel, the enzyme was apparently partially re-solubilized by the concentrated detergent volume. With 1.0% detergent the inclusion volume appeared as a negative peak. This was probably composed of excess sodium trichloroacetate originating from the solubilized enzyme solution.

Figure 5. Plot of Triton X-100 concentration versus degree of solubilization of protein and enzyme. (Buffer consisted of 50 mM HEPES, pH 7.0 and 0.2 mM DTE.)


60.0
55.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Degree of Solubilization (%)
Protein
Enzyme Activity
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Triton X100 (% w/v)

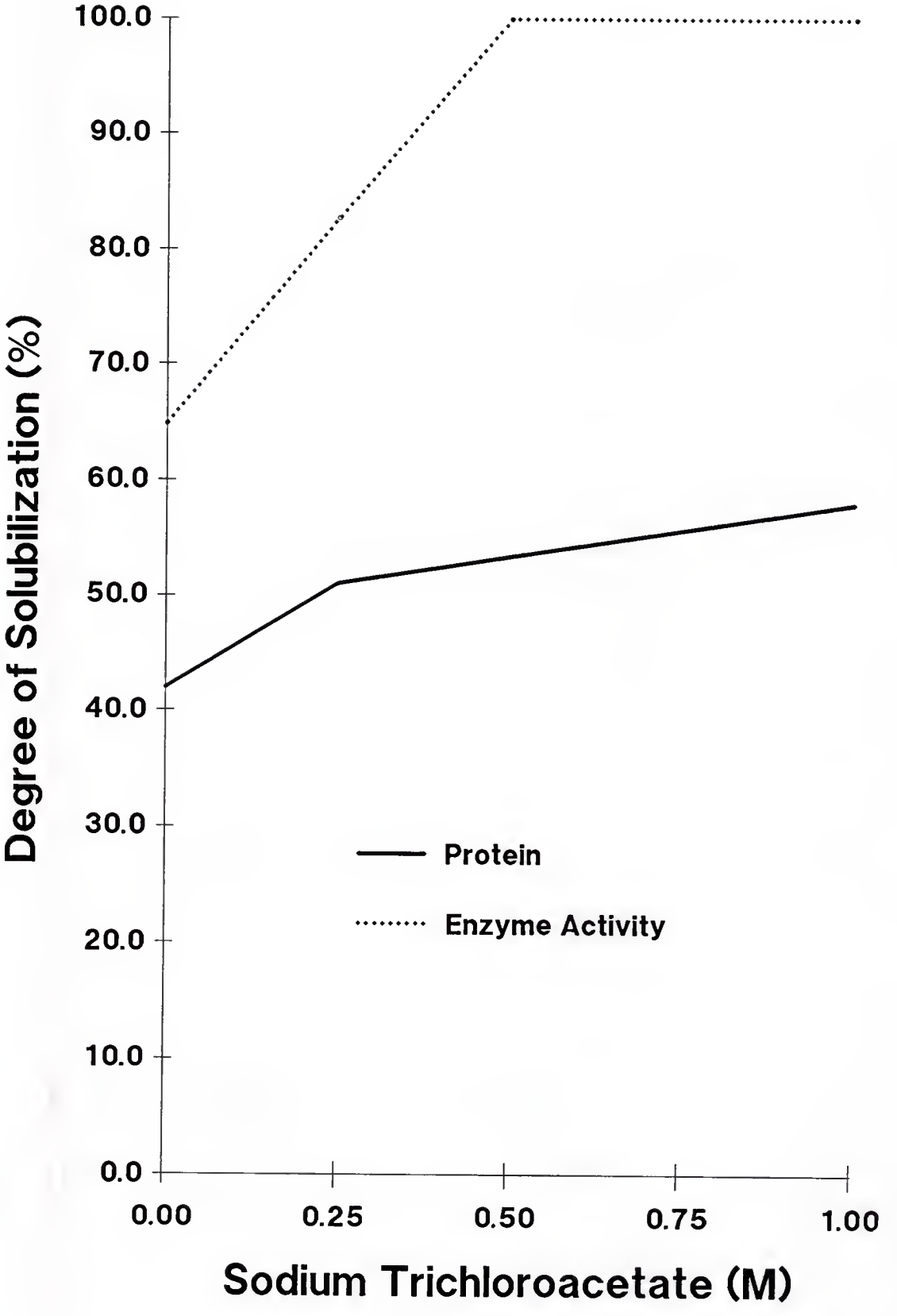
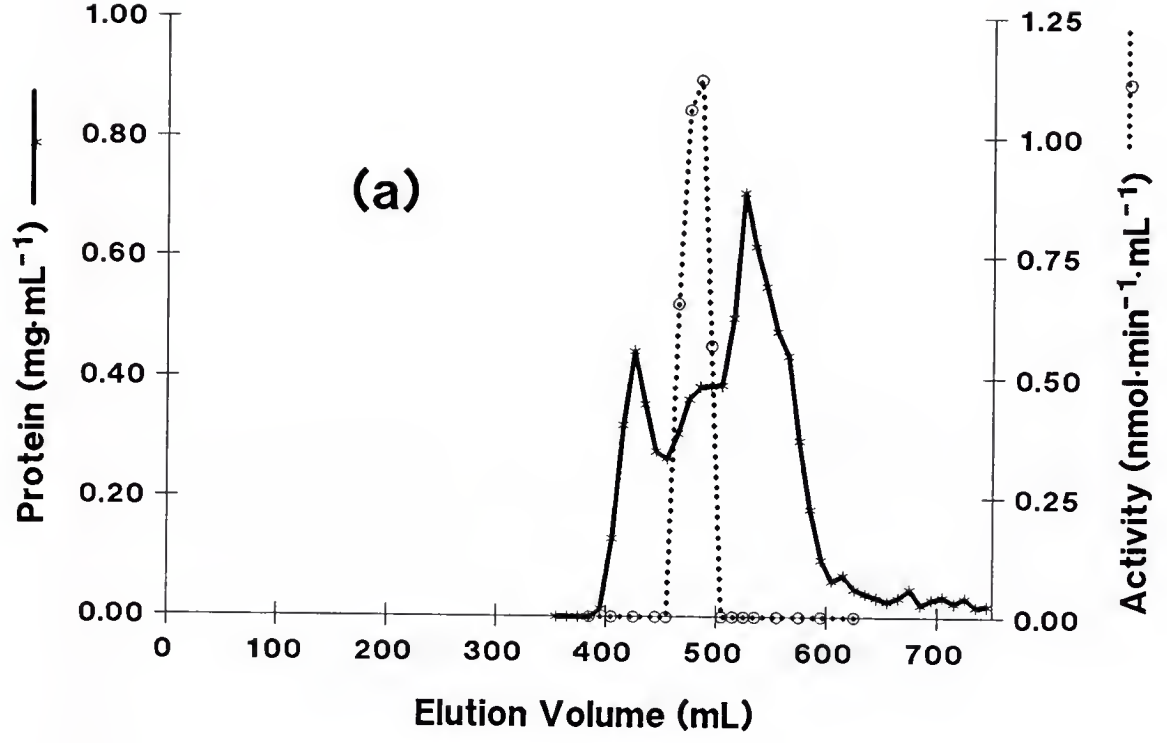
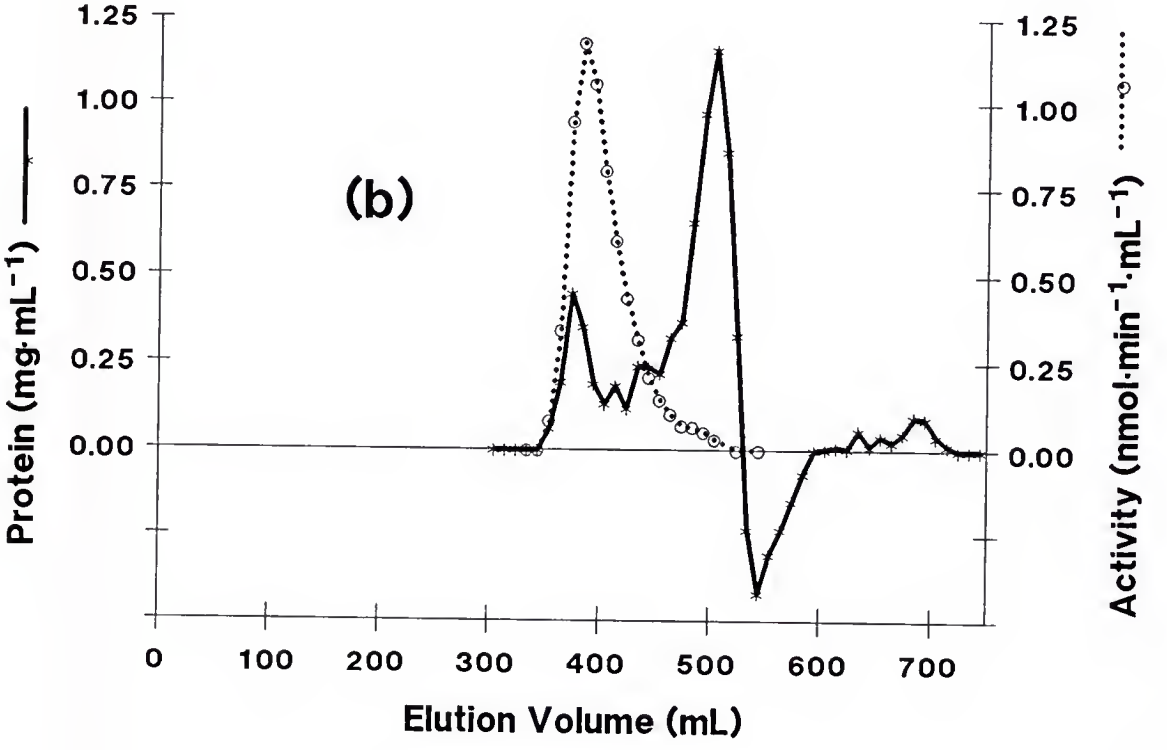

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Degree of Solubilization (%)
Protein
Enzyme Activity
0.00
0.25
0.50
0.75
1.00
Sodium Trichloroacetate (M)

Figure 7. Spectra/Gel AcA 44 gel filtration chromatography of solubilized α-terpineol dehydratase using two concentrations of Triton X-100 in the elution buffer: (a) 0.5% (w/v) Triton X-100 and (b) 1.0% (w/v) Triton X-100. (Elution buffer consisted of 10 mM HEPES, pH 7.0, 0.2 mM DTE, and 0.02% (w/v) sodium azide.)

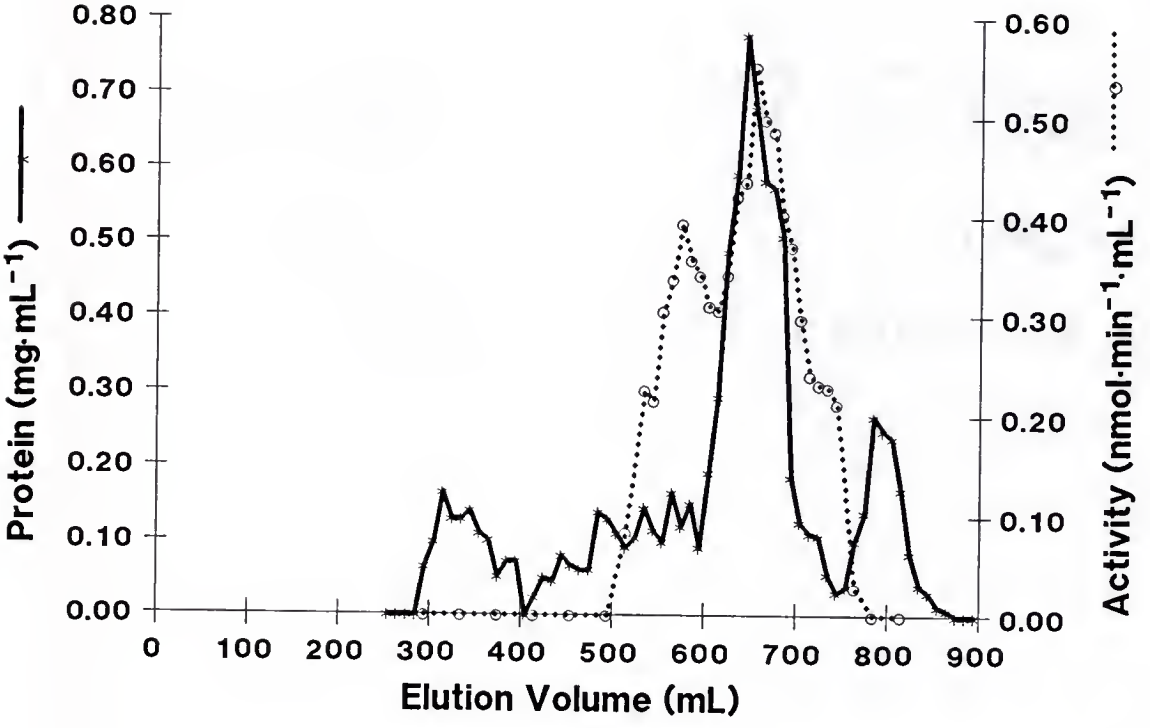

(a)
Protein (mg·mL⁻¹)
Activity (nmol·min⁻¹·mL⁻¹)
Elution Volume (mL)
1.00
0.80
0.60
0.40
0.20
0.00
1.25
1.00
0.75
0.50
0.25
0.00
0
100
200
300
400
500
600
700


(b)
Protein (mg·mL⁻¹)
Activity (nmol·min⁻¹·mL⁻¹)
Elution Volume (mL)
1.25
1.00
0.75
0.50
0.25
0.00
0
100
200
300
400
500
600
700

The purpose of gel filtration chromatography was not only for purification of the enzyme, but also as a criterion to establish the solubility of the enzyme in 1.0% (w/v) Triton X-100. It is generally excepted that an enzyme preparation is "soluble" if it remains in the supernatant solution after centrifugation at 100,000 x g for one or more hours (Penefsky and Tzagoloff, 1971); however, small membrane fragments which do not sediment readily may appear to be soluble. Additional evidence of solubility can be obtained by passage of the enzyme preparation through a gel filtration column (Eiberger and Wasserman, 1987). The enzyme should elute within the linear fractionation range of the column. This is because large insoluble material would elute in the exclusion volume or void volume, unless it precipitates in the column or adsorbs to the gel.

When passed through Spectra/Gel AcA 44 in 1.0% (w/v) Triton X-100 the enzyme eluted in the void volume. The linear fractionation range of AcA 44 is 10,000-130,000 daltons and the exclusion limit is 200,000 daltons. Therefore, the enzyme either eluted as an insoluble enzyme-particulate complex or its molecular weight was larger than the exclusion limit of the gel. When the enzyme was fractionated using Spectra/Gel AcA 22 (with 1.0% (w/v) Triton X-100 in the elution buffer) it eluted as two peaks (Figure 8). The linear fractionation range of AcA 22 is 100,000-1,200,000 daltons and the exclusion limit is 3,000,000 daltons. Both peaks eluted within the linear

Figure 8. Spectra/Gel AcA 22 gel filtration chromatography of solubilized α-terpineol dehydratase. (Elution buffer consisted of 10 mM HEPES, pH 7.0, 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02% sodium azide.)


Protein (mg·mL⁻¹)
Activity (nmol·min⁻¹·mL⁻¹)
Elution Volume (mL)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
700
800
900

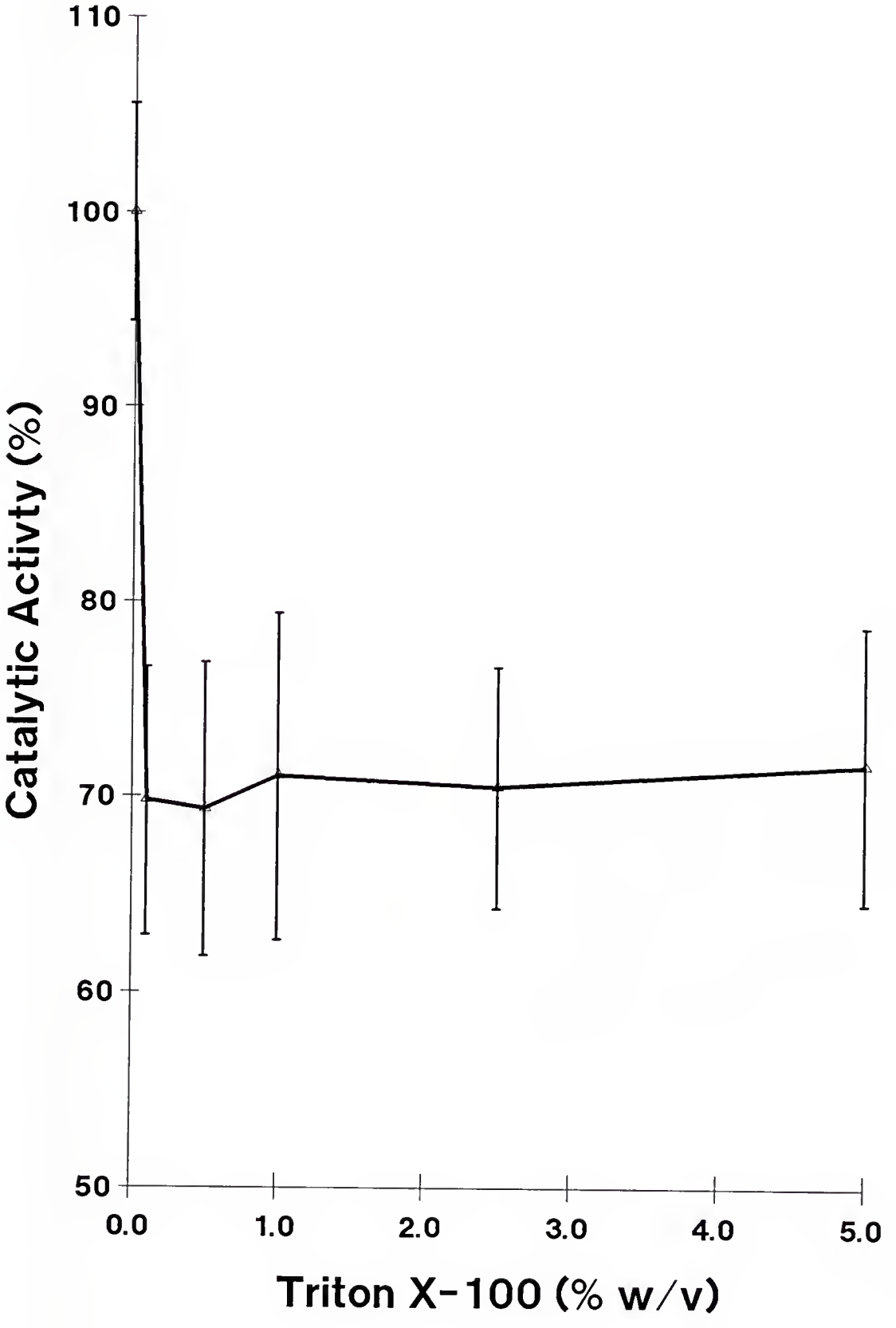
fractionation range of the gel, thereby confirming that the enzyme was soluble in 1.0% (w/v) Triton X-100. One explanation for the occurrence of two activity peaks might be that the enzyme existed as both a native monomer and dimer in the presence of 1.0% (w/v) Triton X-100. Alternatively, the two activity peaks may have been two different enzymes (isozymes).

Partial Purification of Enzyme

Samples from various stages of enzyme fractionation were analyzed for enzyme activity and protein concentration. It was necessary for each sample to contain the same amount of Triton X-100 because concentrations above 0.1% (w/v) had an inhibitory effect on the enzyme (Figure 9). By adjusting the detergent concentration to 0.5% it was possible to compare enzyme activities of different samples.

Protein determinations were of high precision; however, there was a high uncertainty in their accuracy. This was because results obtained using the Coomassie blue protein assay depend on the composition of the protein (Bradford, 1976). Much of the uncertainty could be eliminated by using appropriate blanks to account for the sample matrix. For example, Coomassie blue reacts with Triton X-100 as well as protein. In order to use this method to monitor gel filtration fractions, blanks consisting of the gel filtration buffer were required. The background in the presence of 1.0% detergent was relatively high; however, this method was still preferable to monitoring the

Figure 9. Triton X-100 concentration versus catalytic activity of α-terpineol dehydratase. (Buffer consisted of 10 mM HEPES, pH 7.0. Error bars represent standard deviations, n = 4.)


110
100
90
80
70
60
50
Catalytic Activty (%)
0.0
1.0
2.0
3.0
4.0
5.0
Triton X-100 (% w/v)

absorbance (280 nm) of the gel filtration eluent. Triton X-100 has a very high absorptivity at this wavelength. Uncertainty associated with using bovine serum albumin as the standard could not be eliminated, since this would require using purified enzyme as the standard.

Results of the fractionation of the enzyme using Spectra/Gel AcA 44 chromatography as the final purification step are given in Table 3. The highest degree of purification was obtained after solubilization of the enzyme (5.0 fold). The final degree of purification depended on the amount of particulate-associated enzyme recovered from the 15,000 x g supernatant. Nearly 100% of the enzyme activity was recovered after gel filtration; however, the degree of purification obtained was only about two-fold over the solubilized enzyme. Despite the low purification fold, gel filtration removed much of the pigmented material and appeared to lower the viscosity of the enzyme solution.

Results of the fractionation of the enzyme using Spectra/Gel AcA 22 chromatography as the final purification step are given in Table 4. Again, the highest degree of purification was obtained after solubilization of the enzyme (6.5 fold). The purification factor after gel filtration on AcA 44 could not be accurately determined because the pooled fractions contained an excessively high amount of Triton X-100. This caused a high estimation of the protein concentration. Inflated protein concentration values in turn caused the calculated specific activities to appear

TABLE 3

Fractionation of α-terpineol dehydratase using Spectra/Gel AcA 44 chromatography

Fraction	Volume (mL)	Protein Conc. ($mg \cdot mL^{-1}$)[b]	Activity ($nmol \cdot min^{-1} \cdot mL^{-1}$)[b]	Specific Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)[c]	Percent Recovery	Purification Factor (fold)
Disrupted Cells	194	12.9±0.3	5.1±0.9	0.40±0.07	100	1.00
15,000 x g Supern.	187	11.2±0.4	3.84±0.07	0.342±0.014	72.3	0.86
Enzyme Extract	75.5	7.5±0.6	4.09±0.27	0.55±0.06	31.1	1.37
Solubilized Enzyme	70.0	1.33±0.06	3.69±0.10	2.76±0.16	26.0	6.94
Gel Filtration[a]	270	0.175±0.005	0.92±0.05	5.2±0.3	25.1	13.3

[a]Spectra/Gel AcA 44 chromatography in 10 mM HEPES buffer, pH 7.0 containing 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02% (w/v) sodium azide (fractions from 3 runs, 5 mL each, pooled).

[b]average ± standard deviation (n = 4)

[c]average ± standard deviation (propagated from protein and enzyme concentration data)

TABLE 4

Fractionation of α-terpineol dehydratase using Spectra/Gel AcA 22 chromatography

Fraction	Volume (mL)	Protein Conc. ($mg \cdot mL^{-1}$)[b]	Activity ($nmol \cdot min^{-1} \cdot mL^{-1}$)[b]	Specific Activity ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)[c]	Percent Recovery	Purification Factor (fold)
Disrupted Cells	214	12.6±0.8	5.29±0.06	0.420±0.028	100	1.00
15,000 x g Supern.	200	10.19±0.21	4.50±0.07	0.442±0.011	79.6	1.05
Enzyme Extract	80.0	8.47±0.06	8.00±0.18	0.944±0.022	56.6	2.25
Solubilized Enzyme	75.0	1.178±0.024	7.56±0.24	6.16±0.24	48.1	14.7
Gel Filtration[a]	600	0.178±0.014	0.889±0.017	5.0±0.4	46.9	11.9

[a]Spectra/Gel AcA 22 chromatography in 10 mM HEPES buffer, pH 7.0 containing 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02% (w/v) sodium azide (fractions from 3 runs, 5 mL each, pooled).

[b]average ± standard deviation (n = 4)

[c]average ± standard deviation (propagated from protein and enzyme concentration data)

lower than the actual values. The main disadvantage of AcA 22 gel filtration chromatography was that the total volume of fractions containing enzyme activity was about double that from AcA 44 gel filtration chromatography. This meant additional time was required to concentrate the fractions and reduce the detergent concentration.

Characterization of α-Terpineol Dehydratase

Enzyme-catalyzed dehydration of α-terpineol to limonene could not demonstrated. In fact, no change in limonene concentration could be detected in the assay medium even after 20 hr incubation of the enzyme with 50 mM racemic α-terpineol or 5 mM (4R)-(+)-α-terpineol. The kinetic properties of the enzyme were therefore determined only for the hydration of limonene to α-terpineol. Furthermore, (4R)-(+)-limonene was used as the substrate for all enzyme assays, except for stereospecificity/stereoselectivity studies.

Enzyme Nomenclature

It was decided that the enzyme would be named α-terpineol dehydratase even though the dehydration of α-terpineol to limonene could not be demonstrated. This name was chosen because it was most descriptive of the reaction. It is obvious that dehydration of α-terpineol leads only to limonene. The name limonene hydratase was not chosen because it is ambiguous in regards to which double bond of limonene is hydrated. The name α-terpineol synthase was not chosen because it describes only the product.

Molecular Weight

Initial attempts to determine the native molecular weight of α-terpineol dehydratase involved nondenaturing polyacrylamide gel electrophoresis (ND-PAGE). The method of Doerner and White (1990) was used except that 0.1 or 1.0% (w/v) Triton X-100 was included in all gels and buffers. In 0.1% detergent the sample proteins were unable to migrate through the separating gels. In order to increase the mobility of the proteins, 1.0% detergent was used. Several components of the enzyme solution were partially resolved with ND-PAGE in 1.0% detergent. Gels were composed of 5% or 6% acrylamide (with stacking gels composed of 4% acrylamide). Gels were prepared from an acrylamide stock solution containing 30%T and 2.7%C. It was difficult to distinguish most of the protein bands because the gel backgrounds were very dark due to the high concentration of detergent. No enzyme activity was detected on either gel; therefore it was not possible to identify the enzyme band. It was not surprising that α-terpineol dehydratase could not be separated by ND-PAGE, since the method is generally not applicable to hydrophobic membrane proteins (Tzagoloff and Penefsky, 1971).

The molecular weight of native α-terpineol dehydratase in the presence of 1.0% (w/v) Triton X-100 was determined by gel filtration. A profile for Sepharose CL-6B gel filtration chromatography of α-terpineol dehydratase with 1.0% (w/v) Triton X-100 in the elution buffer is shown in

Figure 10. The linear fractionation range of Sepharose CL-6B is 10,000-4,000,000 daltons. Enzyme activity eluted as two peaks, as was also observed on Spectra/Gel AcA 22. Molecular weights of the two forms of α-terpineol dehydratase were estimated by comparing their V_e/V_o (where V_e is elution volume of sample and V_o is void volume of column) values with a calibration curve of molecular weight versus V_e/V_o for protein standards (Figure 11). Results (Table 5) suggest that α-terpineol dehydratase existed as two forms in 1.0% (w/v) Triton X-100; a monomer with an apparent molecular weight of 94,500 daltons, and a dimer with an apparent molecular weight of 206,500 daltons. Solubilized membrane enzymes often exist as detergent-enzyme complexes (Nalecz et al., 1986). It is possible that molecular weights determined by this technique are those of detergent-enzyme complexes and are not necessarily the actual molecular weights of the enzyme monomer and dimer.

Several enzyme fractions were analyzed by SDS-PAGE (Figure 12) to determine their protein subunit composition. Molecular weights of protein subunits were determined by comparing their migration distances with a calibration curve of molecular weight versus migration distance for protein standards (Figure 13). At least three bands were enriched during the purification of the enzyme, corresponding to polypeptides with molecular weights of 92,000, 38,000, and 28,000 daltons. The 92,000 dalton polypeptide had the highest molecular weight of any other polypeptide in the

Figure 10. Sepharose CL-6B gel filtration chromatography of α-terpineol dehydratase. (Elution buffer consisted of 10 mM HEPES, pH 7.0, 1.0% (w/v) Triton X-100, 0.2 mM DTE, and 0.02% (w/v) sodium azide.)

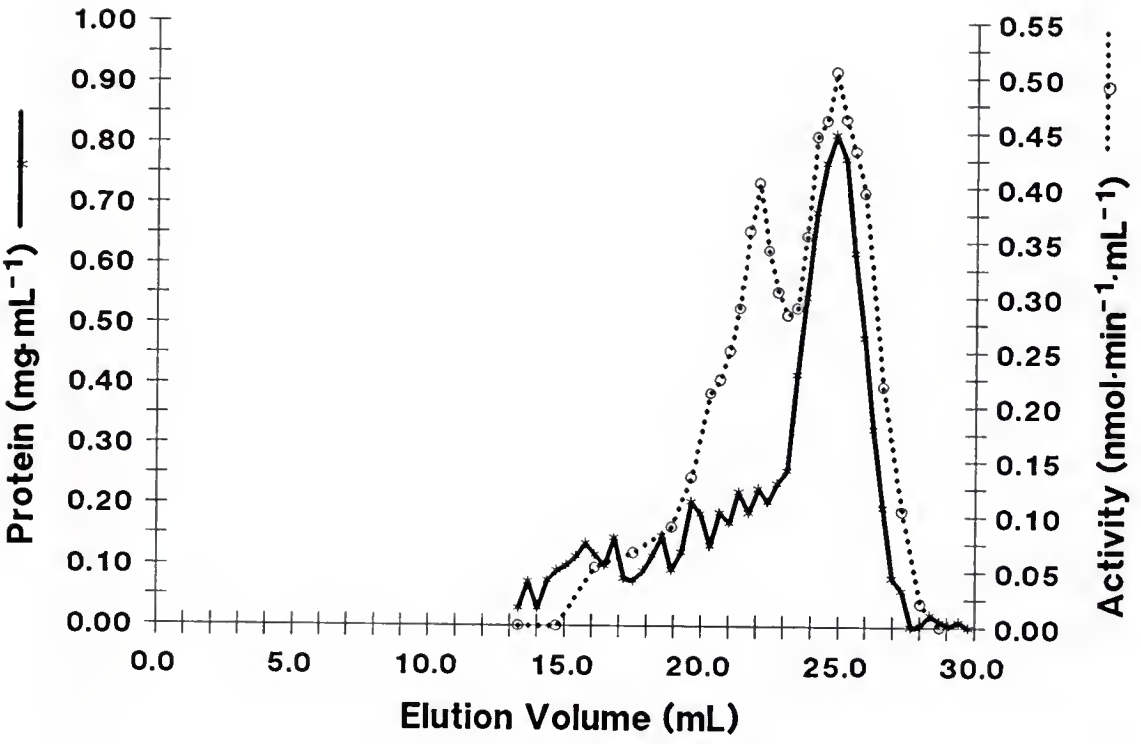

Protein (mg·mL⁻¹)
Activity (nmol·min⁻¹·mL⁻¹)
Elution Volume (mL)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0

Figure 11. Calibration curve for Sepharose CL-6B column. (Values represent means, n = 2.)

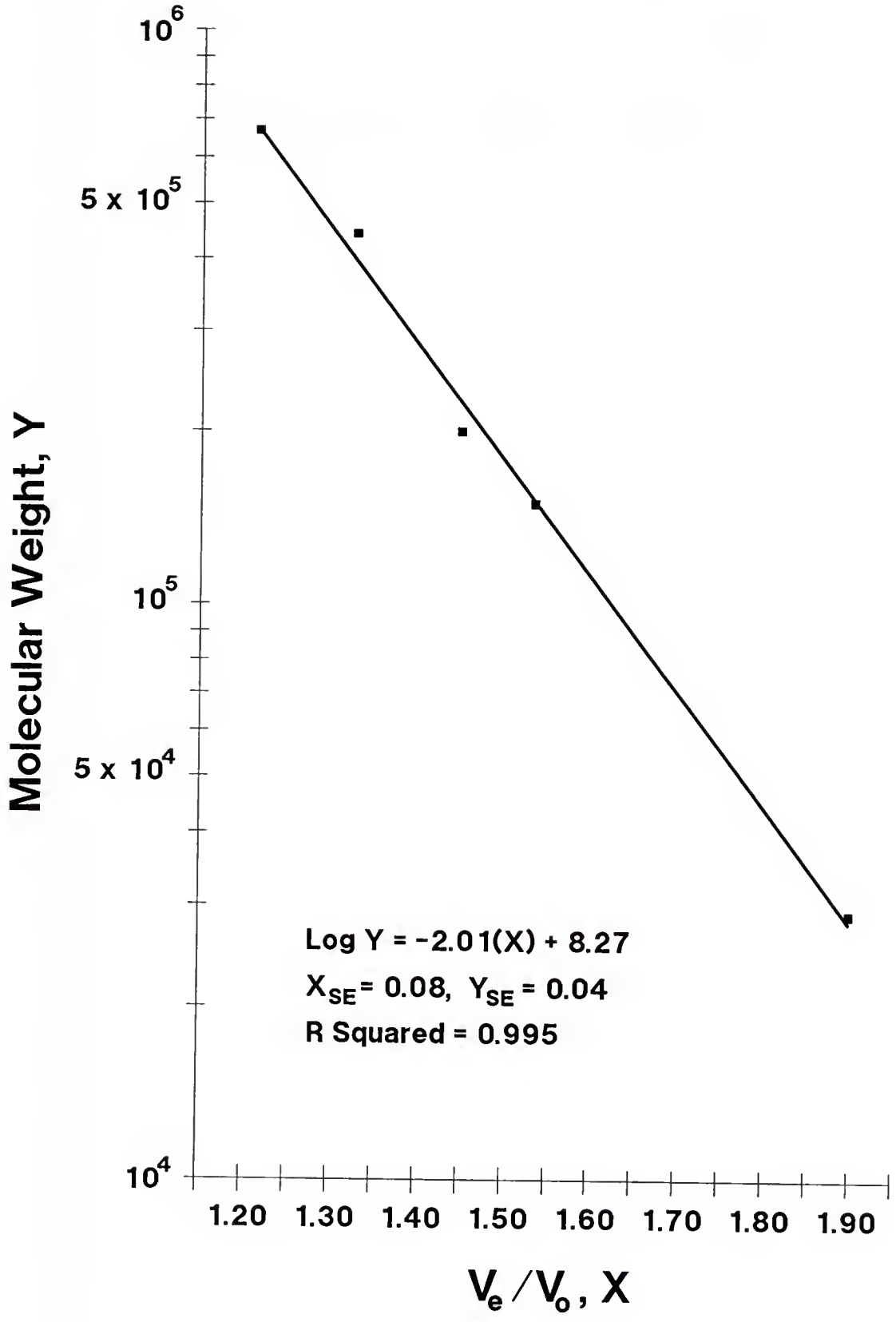

Molecular Weight, Y
10^6
5 x 10^5
10^5
5 x 10^4
10^4
Log Y = -2.01(X) + 8.27
X_SE = 0.08, Y_SE = 0.04
R Squared = 0.995
1.20
1.30
1.40
1.50
1.60
1.70
1.80
1.90
V_e / V_o, X

TABLE 5

Results of the molecular weight determination of native α-terpineol dehydratase in 1.0% (w/v) Triton X-100 by Sepharose CL-6B gel filtration chromatography

Peak Number	V_e/V_o[a]	Molecular Weight (daltons)
1	1.469±0.013[b]	206,500±1800[b]
2	1.638±0.003	94,500±200

[a]V_e = elution volume of sample, V_o = void volume of column

[b]average ± standard deviation (n = 2)

Figure 12. SDS-PAGE gradient (8-18%) gel of enzyme fractions. [Lane 1, 10 μL of 1:100 diluted low molecular weight standards. Lane 2, 10 μL of recovered particulate-associated enzyme (0.25 $mg \cdot mL^{-1}$ protein). Lane 3, 10 μL of solubilized enzyme (0.25 $mg \cdot mL^{-1}$ protein). Lane 4, 10 μL of concentrated AcA 44 gel filtration fractions (0.25 $mg \cdot mL^{-1}$ protein). Lane 5, 10 μL of 1:100 diluted high molecular weight standards. Molecular weight standards were as follows:

a. Myosin (MW = 200,000)
b. *E. coli* ß-galactosidase (MW = 116,250)
c. Rabbit muscle phosphorylase b (MW = 97,400)
d. Bovine serum albumin (MW = 66,200)
e. Hen egg white ovalbumin (MW = 45,000)
f. Bovine carbonic anhydrase (MW = 31,000)
g. Soybean trypsin inhibitor (MW = 21,500)
h. Hen egg-white lysozyme (MW = 14,400).]

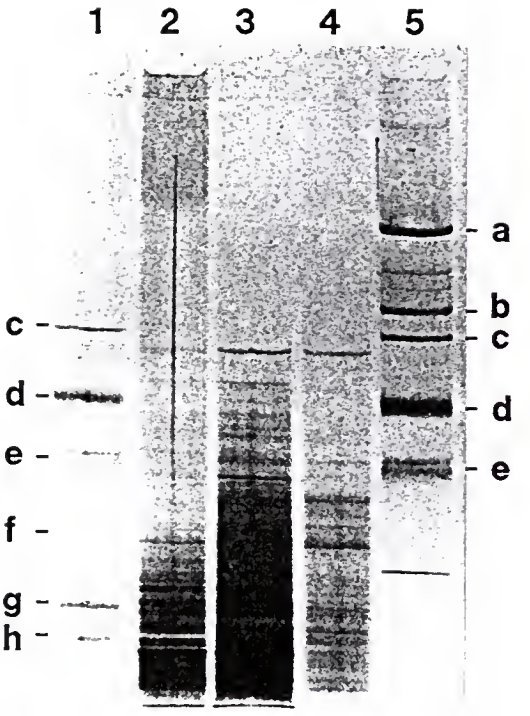
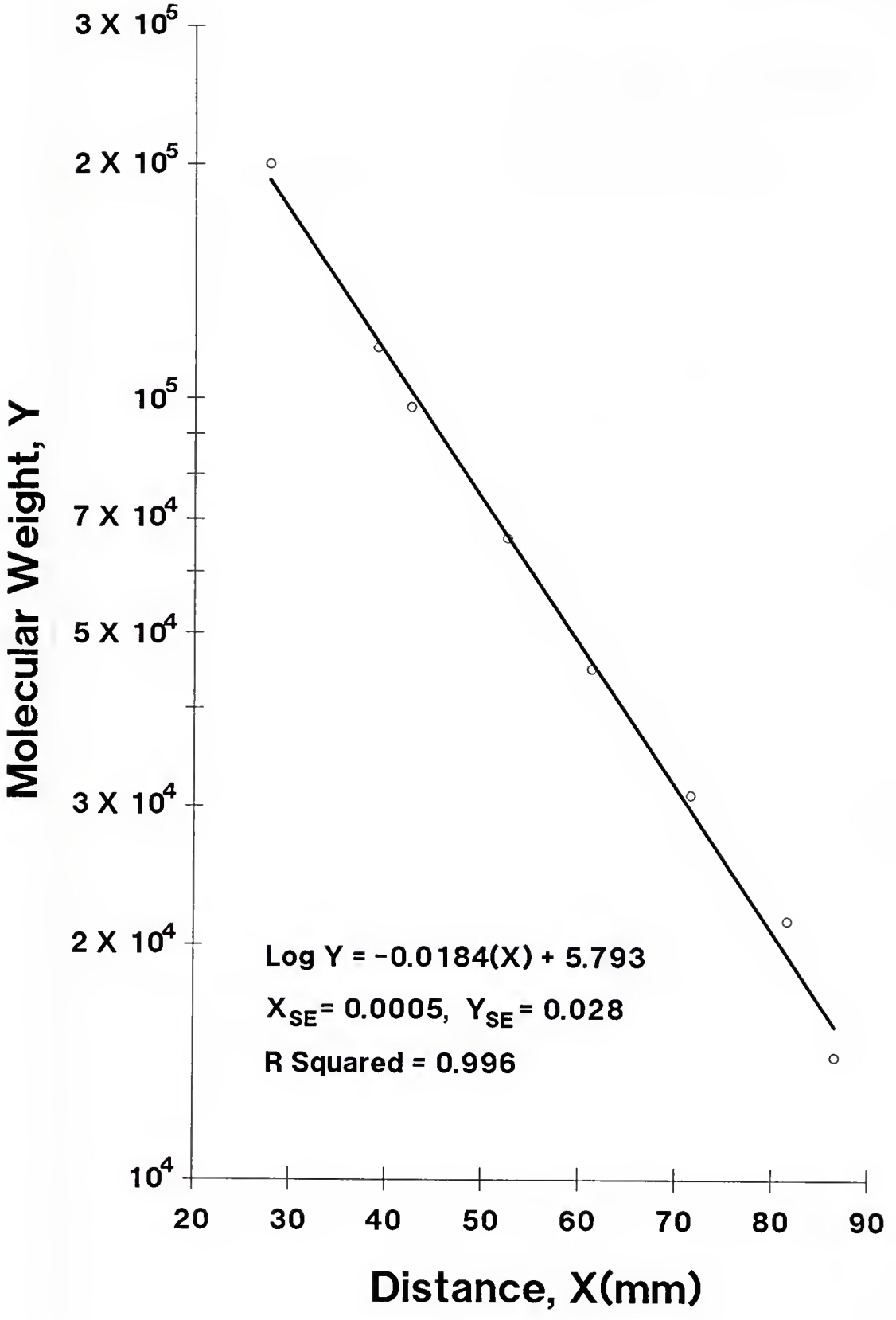

1
2
3
4
5
a
b
c
d
e
c
d
e
f
g
h

Figure 13. Calibration curve for SDS-PAGE gradient (8-18%) gel.


3 X 10^5
2 X 10^5
10^5
7 X 10^4
5 X 10^4
3 X 10^4
2 X 10^4
10^4
Molecular Weight, Y
Log Y = -0.0184(X) + 5.793
X_SE = 0.0005, Y_SE = 0.028
R Squared = 0.996
20
30
40
50
60
70
80
90
Distance, X(mm)

fractions and was essentially the same size as the native α-terpineol dehydratase monomer. This implies that at least some portion of the native monomer did not fractionate into smaller subunits in SDS denaturing conditions. In order to provide conclusive evidence this band represents α-terpineol dehydratase, SDS-PAGE analysis of the purified enzyme would be required.

Isoelectric Point

The isoelectric point (pI) of a protein is the pH at which it has no net charge. One of the easiest and fastest methods for determining the pI of a protein is by isoelectric focusing (IEF), which involves setting up a pH gradient (using ampholytes) and allowing the protein to migrate in an electric field to a point in the system where the pH equals its pI. The pI of α-terpineol dehydratase was determined by agarose IEF in the presence of 1.0% (w/v) Triton X-100 and 2.5% (w/v) ampholytes (Figure 14). Agarose was used as the stabilizing medium instead of polyacrylamide because of its greater porosity. Greater porosity was required so that the high molecular weight proteins could migrate unhindered. The IEF conditions used were suitable for analyzing any protein with a pI between 3 and 10. The pI of α-terpineol dehydratase was determined by comparing its migration distance with a calibration curve of pI versus migration distance for protein standards (Figure 15). Enzyme activity was found throughout most of the gel, with the majority being located in a zone where there were

Figure 14. Agarose IEF gel of partially purified α-terpineol dehydratase in 1.0% (w/v) Triton X-100 and 2.5% (w/v) Servalyte 3-10 ampholytes. [Middle lane, 20 μL (50 μg protein) enzyme solution dialyzed in 1.0% glycine, pH 7.0, containing 1.0% (w/v) Triton X-100. Outside lanes, 10 μL of 1:4 diluted IEF standards. IEF standards were as follows:

a. Phycocyanin (pI = 4.65)
b. ß-Lactoglobulin B (pI = 5.10)
c. Bovine carbonic anhydrase (pI = 6.00)
d. Human carbonic anhydrase (pI = 6.50)
e. Human hemoglobin A (pI = 7.10)
f. Lentil lectin, 3 bands (pI = 7.80, 8.10, 8.20)]

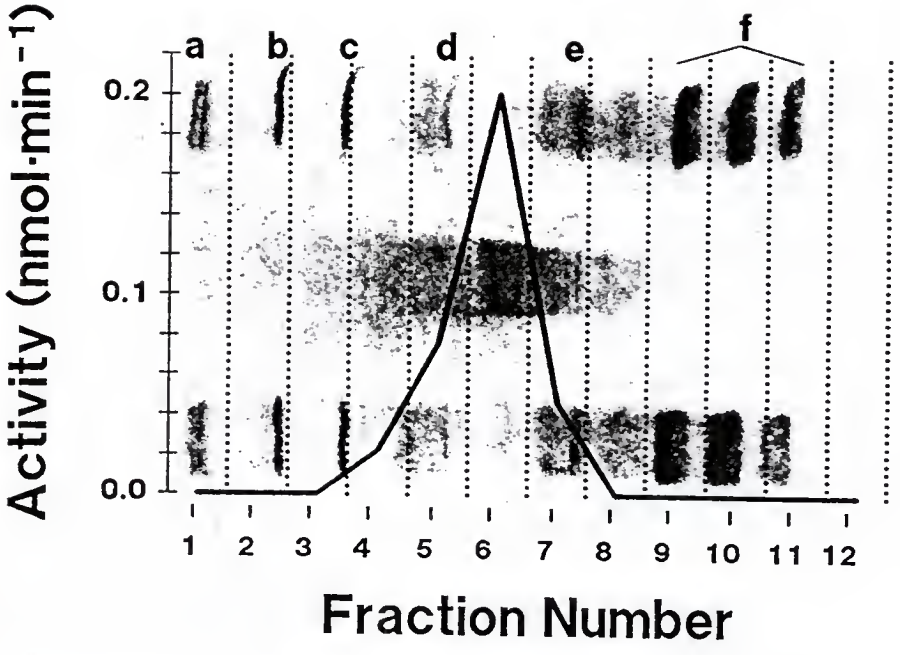
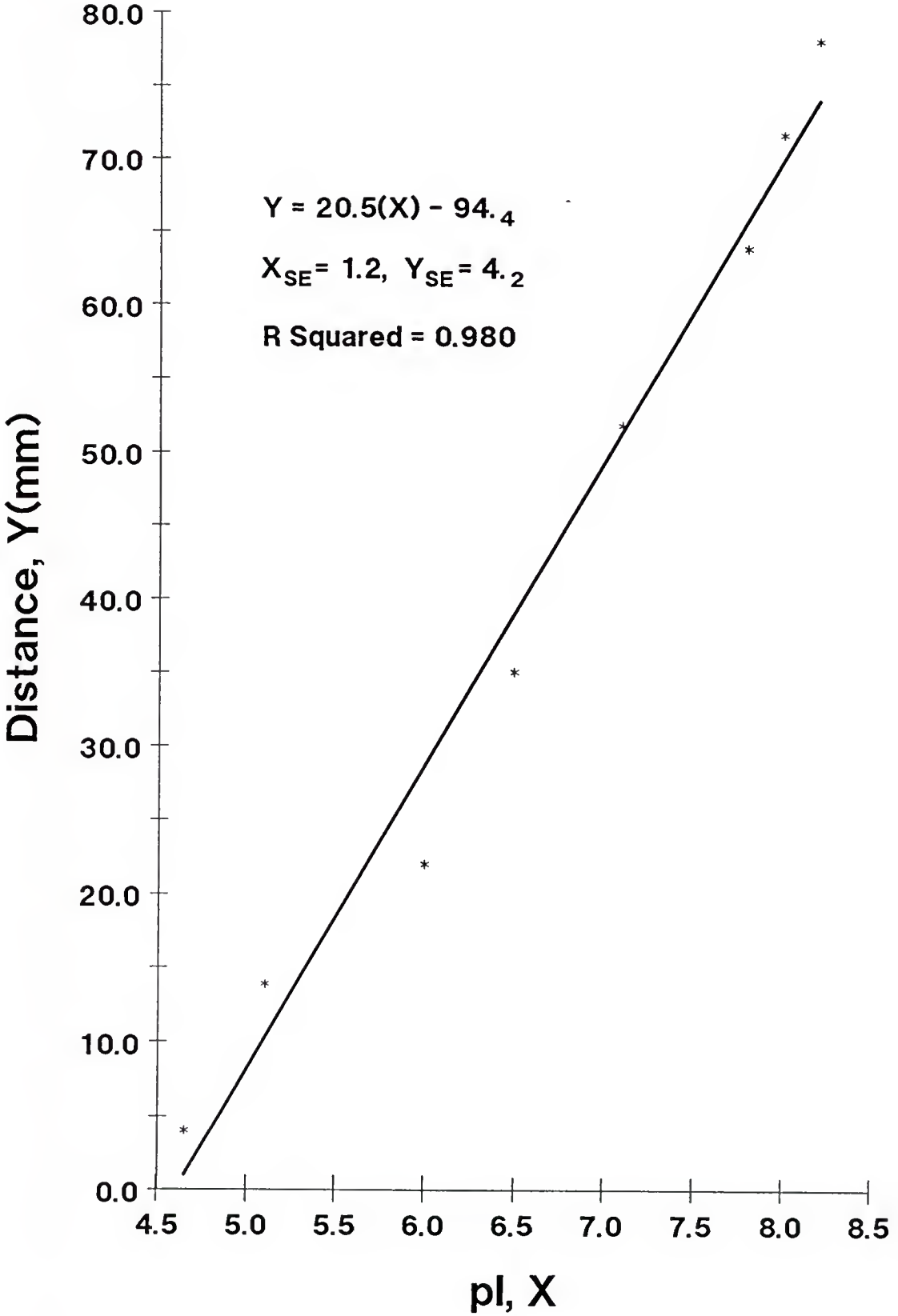

Activity (nmol·min⁻¹)
0.2
0.1
0.0
a
b
c
d
e
f
1
2
3
4
5
6
7
8
9
10
11
12
Fraction Number

Figure 15. Calibration curve for agarose IEF gel. (Values represent means, n = 2.)


80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Distance, Y(mm)
Y = 20.5(X) - 94.4
XSE= 1.2, YSE= 4.2
R Squared = 0.980
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pI, X

several protein bands. This zone actually represented the 0.75 mm gel fraction which had the highest activity. The diffusion of enzyme activity throughout most of the gel was probably due to the association of the enzyme with several ampholyte components, those species having combined pI values different from that of the native enzyme (Scopes, 1987). For the enzyme solution which was dialyzed against 1.0% glycine, the zone of highest activity was composed of proteins with pI values between 6.5 and 6.8. The band of highest intensity in this zone had a pI of 6.6. For the enzyme solution which was dialyzed against distilled water, the zone of highest activity was composed of proteins with pI values between 6.0 and 6.8. The band of highest intensity in this zone also had a pI of 6.6. It was concluded, by comparing the overlap of the zones of highest activity from these two experiments, that the pI of α-terpineol dehydratase was between 6.5 and 6.8. There was not enough evidence to determine which band actually represented α-terpineol dehydratase. Such a determination would require IEF of the purified enzyme.

Activity in Organic Solvents

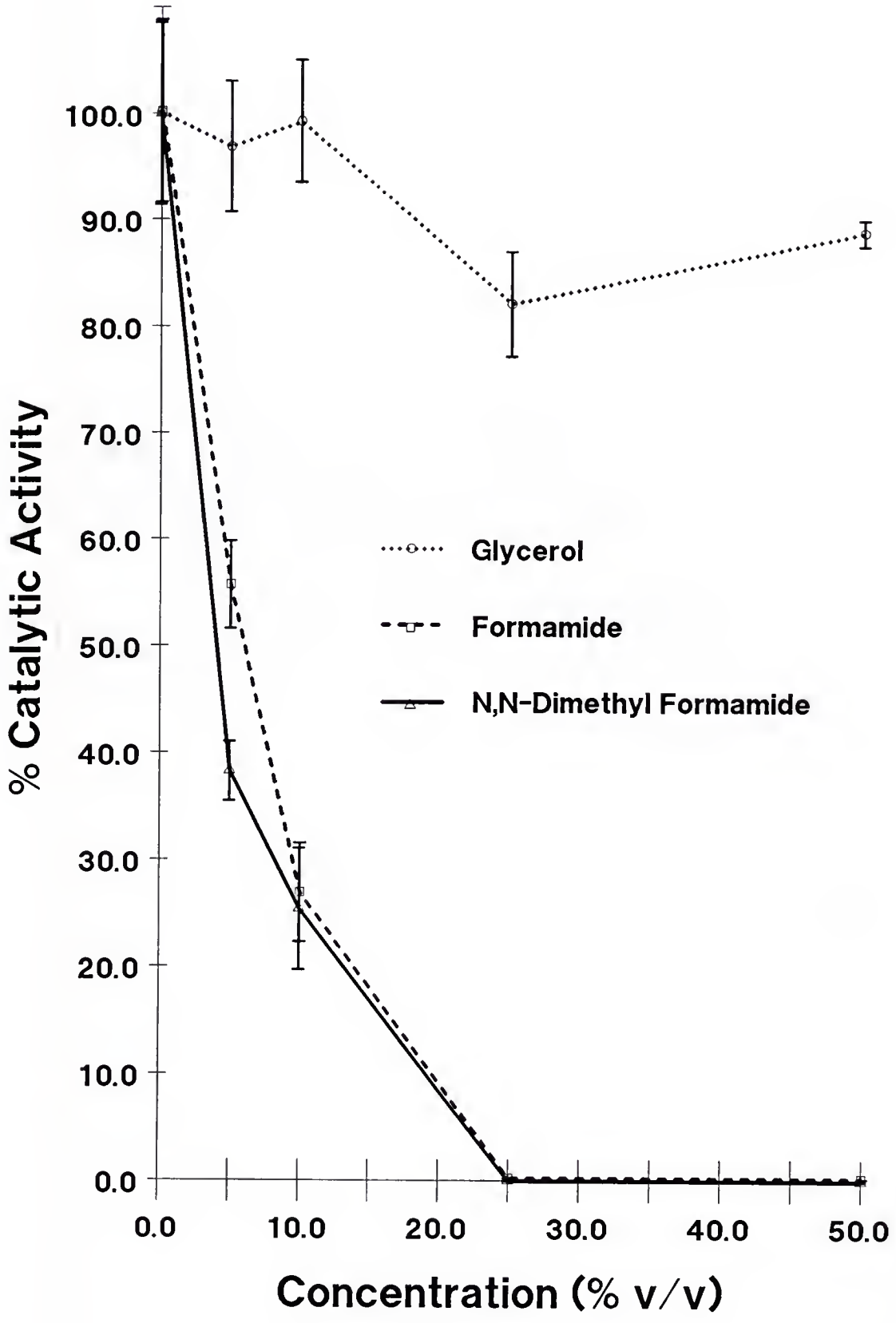
Determination of the kinetic properties of an enzyme becomes complicated when the substrate is insoluble in the medium containing the enzyme. Indeed, classical enzyme kinetic theory assumes that both enzyme and substrate(s) are soluble in the same medium. Therefore, the potential of using water-miscible organic solvents to dissolve limonene in the enzyme reaction medium was examined.

Solvents used in the experiments were carefully selected using the criterion of Khmelnitsky et al. (1988). These researchers stated that hydrophobic interactions play the dominant role in maintaining the catalytically active conformation of enzymes. Thus, the most favored conformation would be produced by solvents that can replace water in the hydration shell of an enzyme molecule without significantly distorting hydrophobic interactions (i.e. solvents would maintain solvophobic interactions).

Ray (1971) classified organic solvents according to their capacity for solvophobic interactions. Solvents were classified into three groups, for example: (i) water, glycerol, ethylene glycol, aminoethanol, and formamide, (ii) methyl formamide and dimethyl formamide; and (iii) methanol, ethanol, and toluene. According to Ray (1971), solvophobic interactions are best realized in solvents of group (i), much less so in solvents of group (ii), and are practically absent in solvents of group (iii). This is because the solvents of group (i) contain at least two groups capable of forming hydrogen bonds. Among the solvents in class (i), water and glycerol have the highest capacity for solvophobic interactions.

Effect of several group (i) and (ii) solvents on α-terpineol dehydratase activity is shown in Figure 16. The effect of glycerol was examined for comparison purposes only, since limonene is scarcely more soluble in glycerol than in water. α-Terpineol dehydratase activity decreased

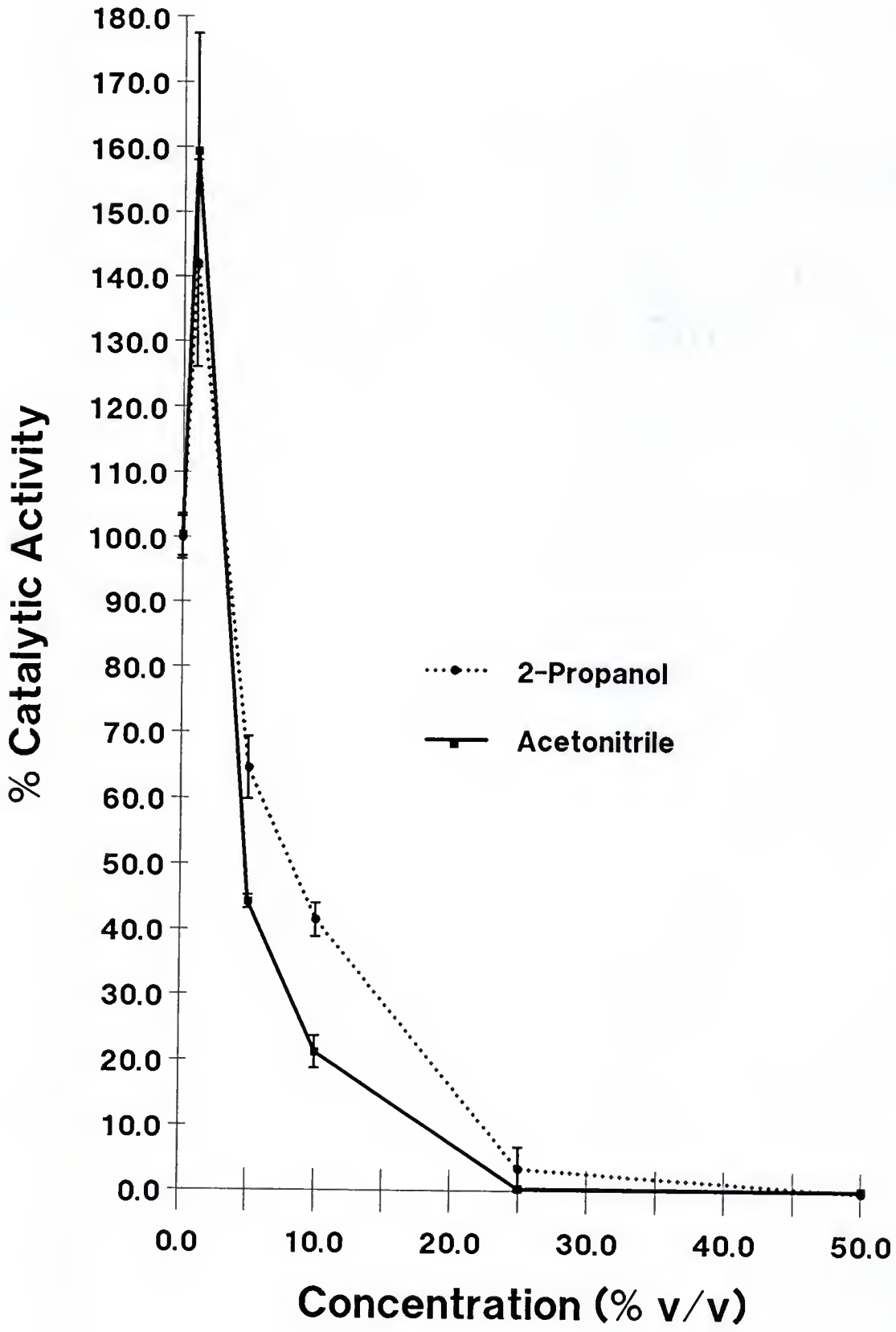
Figure 16. Effect of glycerol, formamide, and N,N-dimethylformamide concentration on the activity of α-terpineol dehydratase. (Error bars represent standard deviations, n = 4.)


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Catalytic Activity
Glycerol
Formamide
N,N-Dimethyl Formamide
0.0
10.0
20.0
30.0
40.0
50.0
Concentration (% v/v)

only slightly in the presence of glycerol, even at concentrations as high as 50% (v/v). Formamide and dimethylformamide effectively lowered the activity, with total inactivation of the enzyme occurring at solvent concentrations above 25% (v/v). Since water is one of the substrates of α-terpineol dehydratase, it was originally thought that the slight decrease in activity observed in 25 or 50% (v/v) glycerol was due to a decrease in the water activity and subsequent shift in equilibrium of the reaction. α-Terpineol dehydratase did not catalyze the reverse reaction under these conditions (using 50 mM racemic α-terpineol as substrate); therefore the decrease was probably not due to a shift in equilibrium. The apparent decrease in activity may have been due to diffusion limitations caused by the high viscosity of the glycerol solutions. Alternatively, the higher concentrations of glycerol may have caused some technical problems, such as a decrease in the recovery of α-terpineol during hexane extraction from the assay solutions.

Effects of the group (iii) solvents, 2-propanol, and acetonitrile, on the α-terpineol dehydratase activity were similar to those observed for formamide and dimethyl-formamide (Figure 17), except that there was an increase in activity in 1.0% (v/v) solvent. The apparent enzyme activation was probably due to increased dispersion of the limonene in the medium. Low activity of α-terpineol dehydratase at the higher solvent concentrations could have

Figure 17. Effect of 2-propanol and acetonitrile concentration on the activity of α-terpineol dehydratase. (Error bars represent standard deviations, n = 4.)

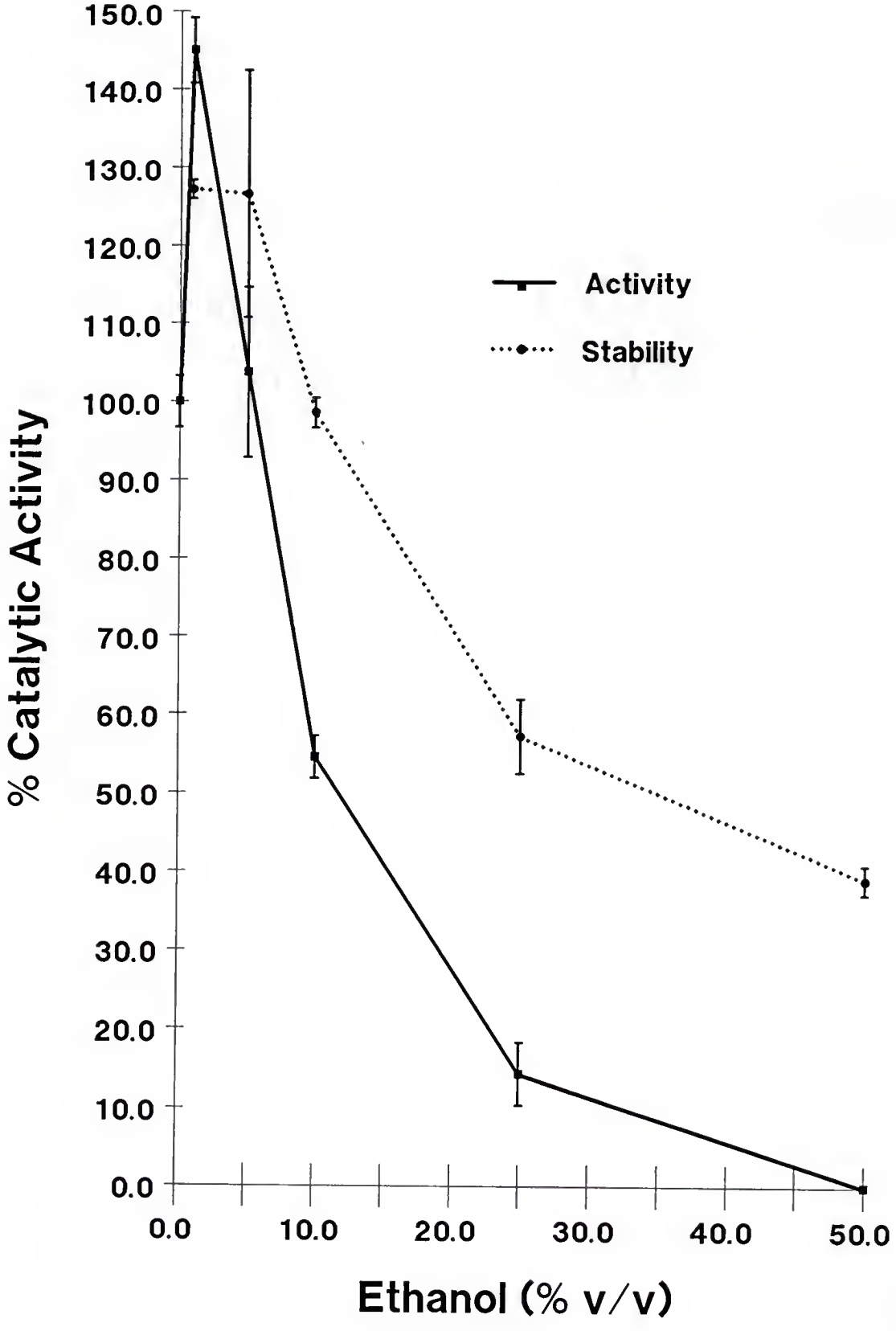

180.0
170.0
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Catalytic Activity
2-Propanol
Acetonitrile
0.0
10.0
20.0
30.0
40.0
50.0
Concentration (% v/v)

been due to irreversible denaturation or a reversible phenomenon such as conformational changes.

In order to elucidate the mechanism, the activity and stability of the enzyme was determined in various concentrations of ethanol (Figure 18). The stability of the enzyme was determined by evaporating the ethanol using a stream of nitrogen and then measuring the residual activity. The effect of ethanol on the activity of α-terpineol dehydratase was essentially the same as that observed for the other group (iii) solvents, 2-propanol and acetonitrile. The stability curve showed that even at 50% (v/v) ethanol not all the enzyme was denatured. α-Terpineol dehydratase did not catalyze the reverse reaction under these conditions (using 50 mM racemic α-terpineol as substrate). Therefore, the decrease in activity at higher ethanol concentrations was probably due to a conformational change (with some degree of denaturation occurring) rather than a shift in equilibrium. It was not possible to remove all of the ethanol from the solutions because of ethanol-water azeotrope formation. This might account for the excessively high residual activities observed for 1 and 5% ethanol.

Effects of various nonpolar solvents on α-terpineol dehydratase activity were similar to the effects observed with water-miscible solvents. Enzyme activity was assayed in 1:1 mixtures consisting of nonpolar solvent plus aqueous enzyme solution. Emulsions were broken by the addition of ethanol and the products analyzed by GC. No activity was

Figure 18. Effect of ethanol concentration on activity and stability of α-terpineol dehydratase. Activity curve: activity versus ethanol concentration. Stability curve: activity after removal of indicated ethanol concentration. (Error bars represent standard deviations, n = 4.)


150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Catalytic Activity
Activity
Stability
0.0
10.0
20.0
30.0
40.0
50.0
Ethanol (% v/v)

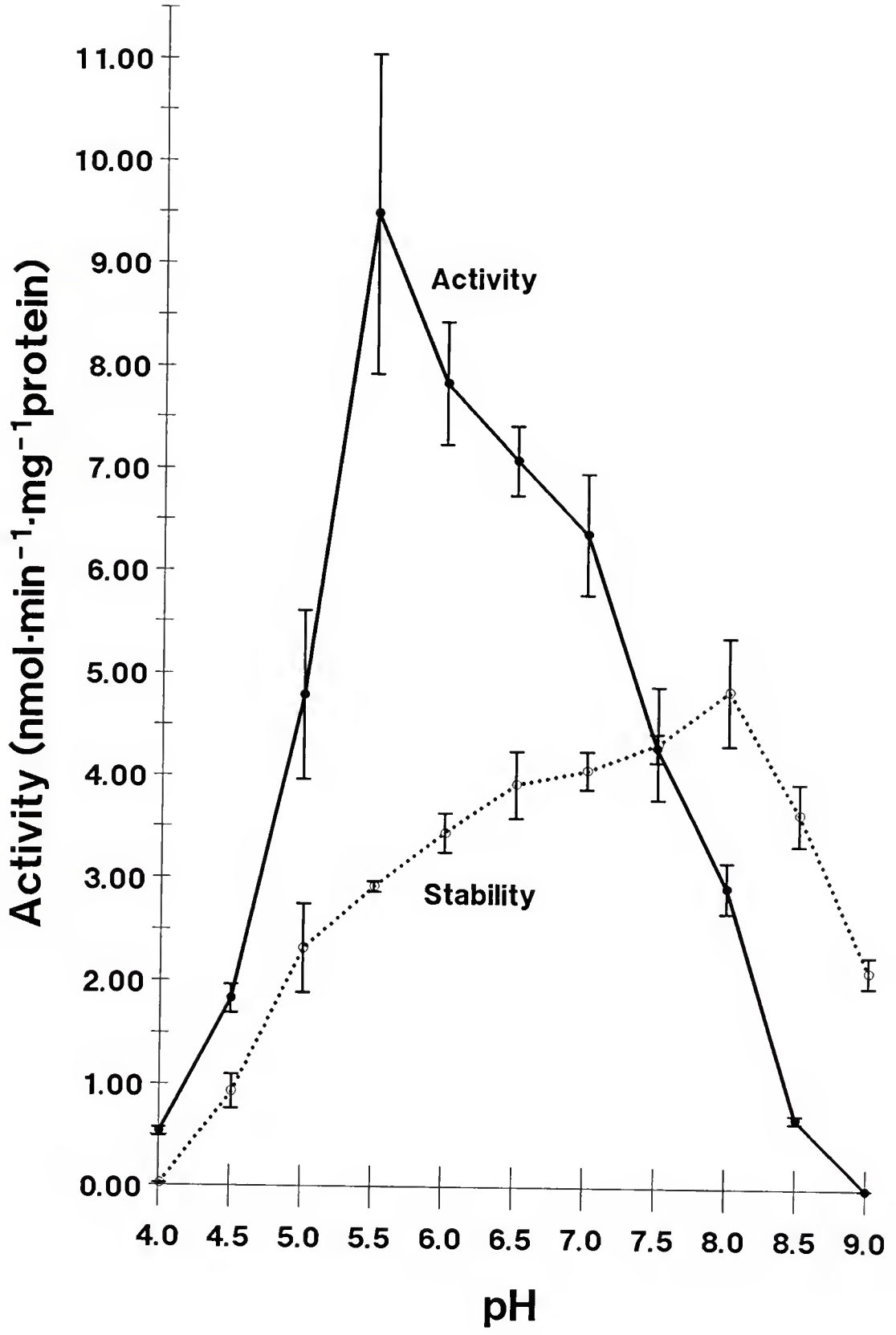
detected in any of the solvents tested: n-hexane, diethyl ether, dichloromethane, chloroform, and limonene. In fact, enzyme reactions could be stopped by addition of equal volumes of nonpolar solvents to enzyme solutions. The high instability of α-terpineol dehydratase in organic solvents should not necessarily eliminate it from being used in non-aqueous bio-catalytic systems. Techniques such as enzyme fixation (immobilization) or spatial separation of the enzyme from the organic solvent could be used to increase enzyme stability (Brink et al., 1988; Khmelnitsky et al., 1988; Klibanov, 1986; and Zaks and Klibanov, 1988). Use of such techniques should be preceded by preliminary characterization of the kinetic properties of α-terpineol dehydratase in a less complex system.

Kinetic properties of α-terpineol dehydratase were determined in aqueous medium containing 0.1% (w/v) Triton X-100. Detergent was included in the medium to maintain the solubility of the enzyme. α-Terpineol dehydratase activity in 0.1% detergent was less sensitive to small differences in detergent concentration than at very low detergent concentrations (see Figure 9). Detergent concentrations higher than 0.1% were not used due to difficulty in breaking the hexane emulsion at the higher concentrations.

pH Optimum and Stability

The pH profile of α-terpineol dehydratase in 10 mM MES, 10 mM BIS-TRIS PROPANE buffer containing 0.1% (w/v) Triton X-100 is shown in Figure 19. The apparent pH optimum of

Figure 19. Effect of pH on activity and stability of α-terpineol dehydratase. Activity curve: activity versus pH. Stability curve: activity at pH 7.0 after 25°C pre-incubation of the enzyme at indicated pH values. (Buffer consisted of 10 mM MES, 10 mM BIS-TRIS PROPANE and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)


Activity (nmol·min⁻¹·mg⁻¹protein)
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Activity
Stability
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

α-terpineol dehydratase occurred at pH 5.5, while the apparent stability optimum occurred at pH 8.0. The pH and stability optima of an enzyme depend on several factors, including: temperature, ionic strength, nature of the buffer, concentration of preservatives, and concentration of the enzyme as well as other proteins (Segel, 1976; Whitaker, 1972). In general, proteins are least stable at pH values near their isoelectric points (Scopes, 1987). Therefore, the higher stability at pH 8.0 was not surprising since this pH is somewhat higher than the pI (6.5-6.8) of α-terpineol dehydratase.

The low activity observed below pH 5.0 was probably due to enzyme inactivation rather than the formation of an improper ionic form of the enzyme, since the enzyme was very unstable below this pH. At the pH of highest enzyme stability, the activity was about one-third as high as that observed at the apparent pH optimum. It is apparent that a slightly acidic environment increased the activity of α-terpineol dehydratase, at the same time lowering its stability. From these results it was decided that all other kinetic experiments would be performed at pH 7.0, since at this pH both the activity and stability of α-terpineol dehydratase were reasonably high.

Temperature Optimum and Stability

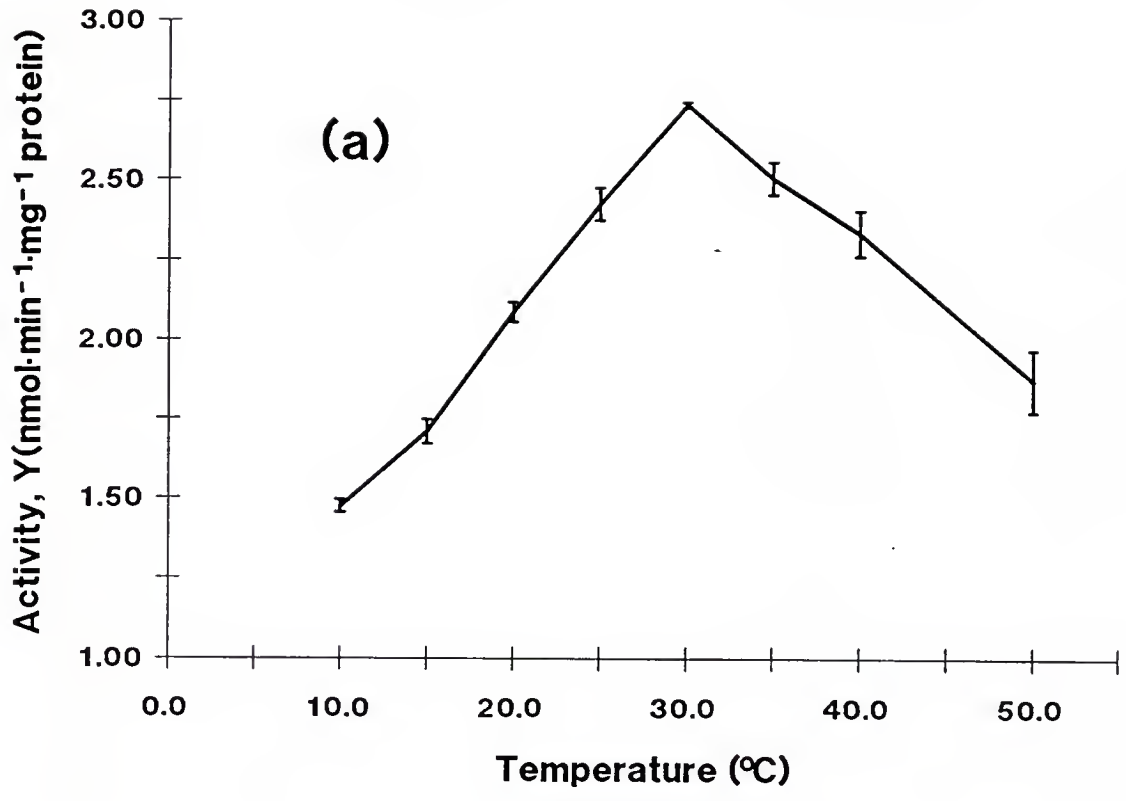
Effect of temperature on the activity of α-terpineol dehydratase in 10 mM HEPES buffer, pH 7.0 containing 0.1% (w/v) Triton X-100 is shown in Figure 20a. The apparent

temperature "optimum" occurred at 30°C. The true optimum is the maximum temperature at which the enzyme exhibits a constant activity over a time of at least as long as the assay time (Segel, 1976). This was determined by pre-incubating the enzyme at various temperatures for the same amount of time as the assay time and then assaying for activity at 20°C. Results of this experiment are shown in Figure 21. The enzyme appeared to be completely stable to 25°C, but began to denature at 30°C. Therefore the true temperature "optimum" of the α-terpineol dehydratase under these conditions was 25°C instead of 30°C.

Some correlation may be made between the apparent molecular weight of α-terpineol dehydratase and its low heat stability. High molecular weight proteins with complex structures are generally more heat labile than low molecular weight proteins composed of single polypeptides with disulfide bonds (Segel, 1976; and Whitaker, 1972). Temperature stability of proteins is also a function of a number of other factors, including the pH and ionic strength of the medium.

Temperature-activity data obtained from 10-25°C were used to estimate the Arrhenius activation energy (E_a) of α-terpineol dehydratase (Figure 20b). Average E_a from duplicate determinations was 21.6 ± 2.9 $kJ \cdot mol^{-1}$. The E_a of α-terpineol dehydratase falls within the range of E_a values (8-50 $kJ \cdot mol^{-1}$) reported for other enzymes (Whitaker, 1972; White et al., 1964). Activity-temperature data used to

Figure 20. The effect of temperature on the activity of α-terpineol dehydratase: (a) plot of activity versus temperature and (b) Arrhenius plot of 10-25°C activity-temperature data. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4)


(a)
Activity, Y(nmol·min⁻¹·mg⁻¹ protein)
3.00
2.50
2.00
1.50
1.00
0.0
10.0
20.0
30.0
40.0
50.0
Temperature (°C)

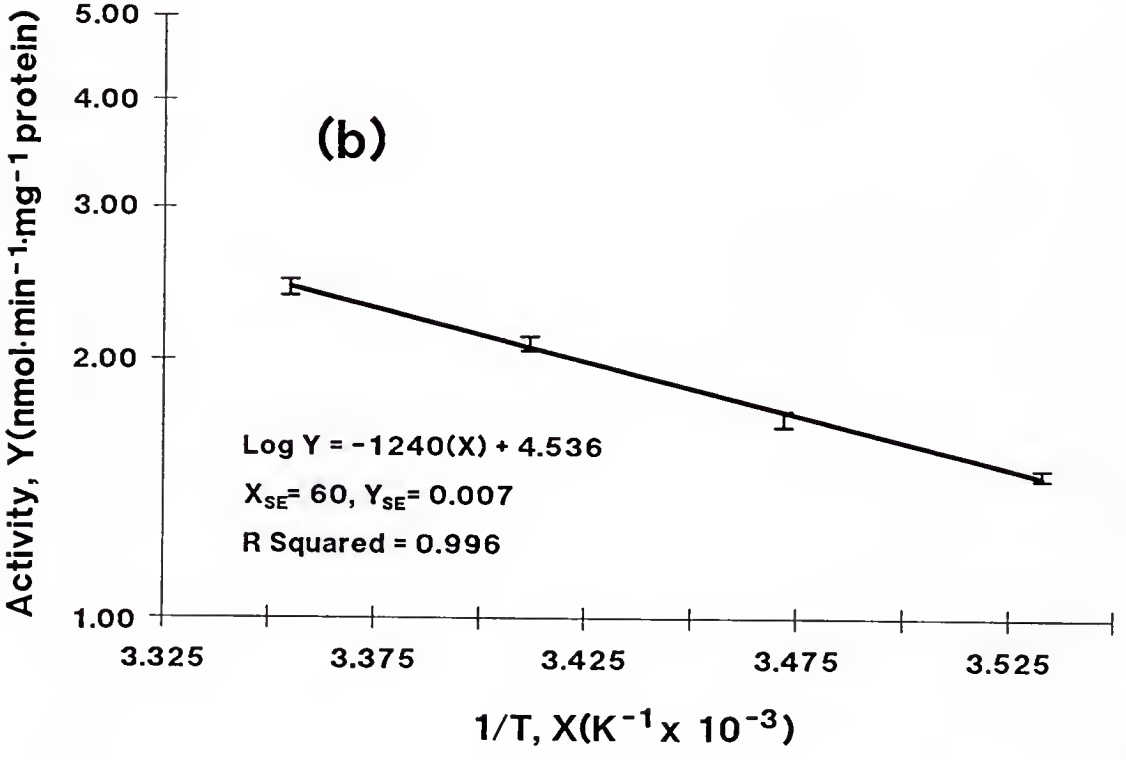
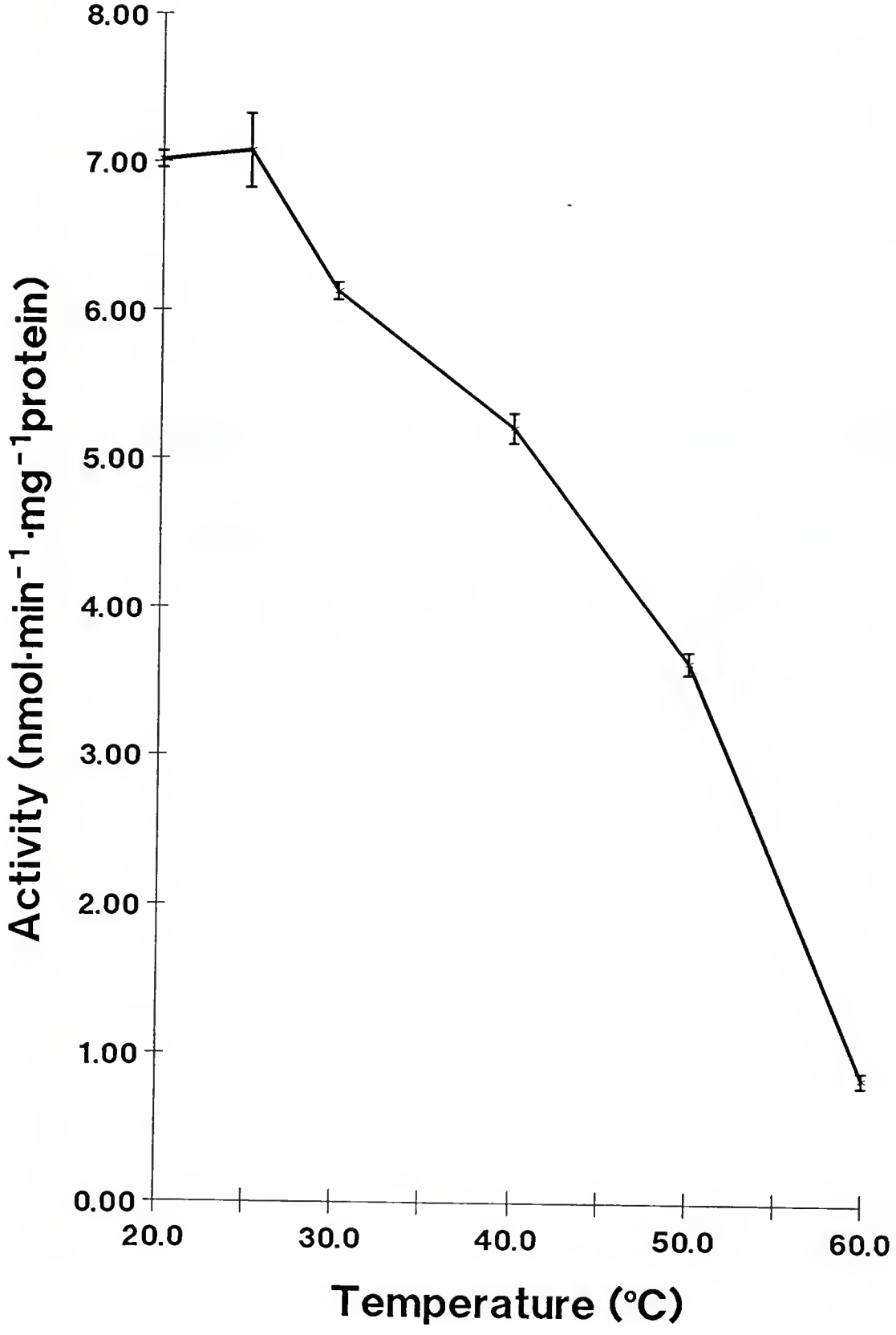

(b)
Activity, Y(nmol·min⁻¹·mg⁻¹ protein)
5.00
4.00
3.00
2.00
1.00
Log Y = -1240(X) + 4.536
X_SE= 60, Y_SE= 0.007
R Squared = 0.996
3.325
3.375
3.425
3.475
3.525
1/T, X(K⁻¹ x 10⁻³)

Figure 21. Temperature stability of α-terpineol dehydratase: activity at 20°C after pre-incubating enzyme at indicated temperatures for 2 min. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)


8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Activity (nmol·min⁻¹·mg⁻¹protein)
20.0
30.0
40.0
50.0
60.0
Temperature (°C)

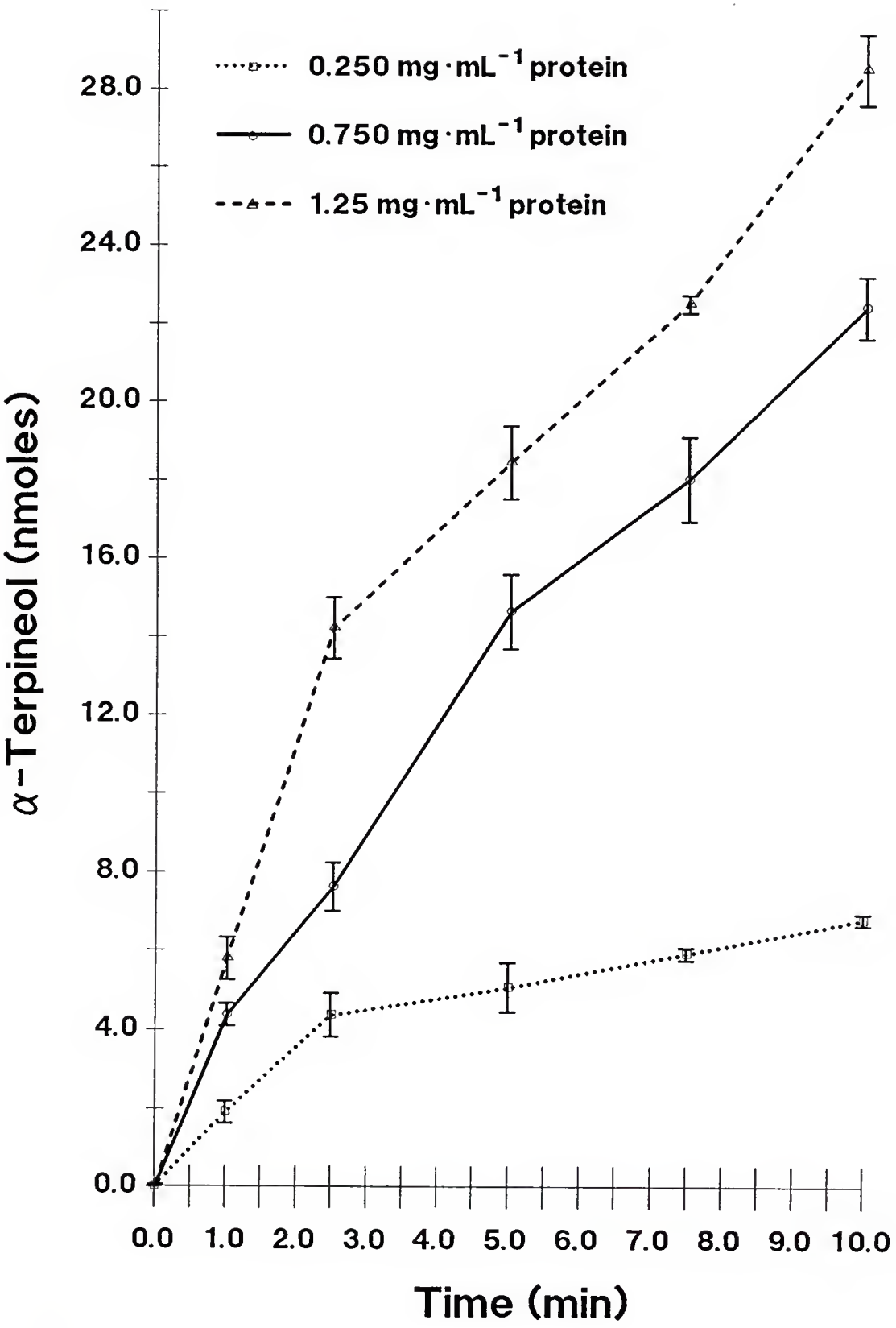
estimate E_a assume that the enzyme was functioning at maximum activity or velocity (V_{max}). However, this was probably not the case. Because K_m varies with temperature, it should not be assumed that a given concentration of substrate was saturating at all temperatures. Ideally, E_a should be estimated from V_{max} values determined at each temperature.

The average Q_{10} for the temperature range 10-25°C was 1.37 ± 0.07. The Q_{10} of α-terpineol dehydratase was smaller than Q_{10} values for most chemical and enzymatic reactions, which generally fall in range from 2-3 (Whitaker, 1972). Lower Q_{10} indicates that activity of α-terpineol dehydratase was not as sensitive to increases in temperature as some other enzymes.

Initial Velocity

Since enzyme reaction velocity varies with the substrate concentration, assay times should be short enough to insure that only a small fraction of the substrate is utilized. Appearance of α-terpineol as a function of time for three concentrations of α-terpineol dehydratase is shown in Figure 22. Rate of α-terpineol formation was constant (zero order) for all three concentrations of enzyme up to 2.5 min. Therefore, assay times less than 2.5 min should give linear responses for "catalytic" concentrations of enzyme. Unless otherwise stated, enzyme assays were conducted using 2 min incubation times. Initial velocity as a function of concentration of α-terpineol dehydratase is

Figure 22. Formation of α-terpineol versus time for three concentrations of α-terpineol dehydratase at 25°C and 50 mM limonene. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)


0.250 mg·mL⁻¹ protein
0.750 mg·mL⁻¹ protein
1.25 mg·mL⁻¹ protein
α-Terpineol (nmoles)
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
Time (min)

shown in Figure 23. α-Terpineol dehydratase was present in "catalytic" amounts at all protein concentrations examined.

Effect of Triton X-100 on K_m and V_{max}

Effect of varying the Triton X-100 concentration on Henri-Michaelis-Menten (v versus [S], where v is activity or velocity and [S] is limonene concentration) curves is shown in Figure 24. All curves appear to follow Henri-Michaelis-Menten kinetics (i.e., their curvature is constant); however, the activity was inhibited by the presence of 0.1 and 0.5% (w/v) Triton X-100. Kinetic parameters, K_m and V_{max}, were determined using Lineweaver-Burk double reciprocal (1/v versus 1/[S]) plots (Figure 25). Quantitative results are presented in Table 6. The double reciprocal plot is based on the rearrangement of the Henri-Michaelis-Menten equation to a linear (y = mx + b) form: $1/v = K_m/V_{max}(1/[S]) + 1/V_{max}$. The K_m was determined from the slope (K_m/V_{max}) and the V_{max} from the intercept of the 1/v axis ($1/V_{max}$). The K_m and V_{max} determined under the conditions of this study are apparent values, since the enzyme and limonene were not actually soluble in the medium but instead were suspended in detergent micelles. As a consequence of including Triton X-100 in the medium, the V_{max} decreased and the K_m increased.

The effect of Triton X-100 was uncharacteristic of classical enzyme inhibition (e.g., competitive, noncompetitive, uncompetitive, or irreversible inhibition), since none of these result in a simultaneous decrease in V_{max}

Figure 23. Initial velocity as a function of α-terpineol dehydratase concentration at 25°C and 50 mM limonene. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)

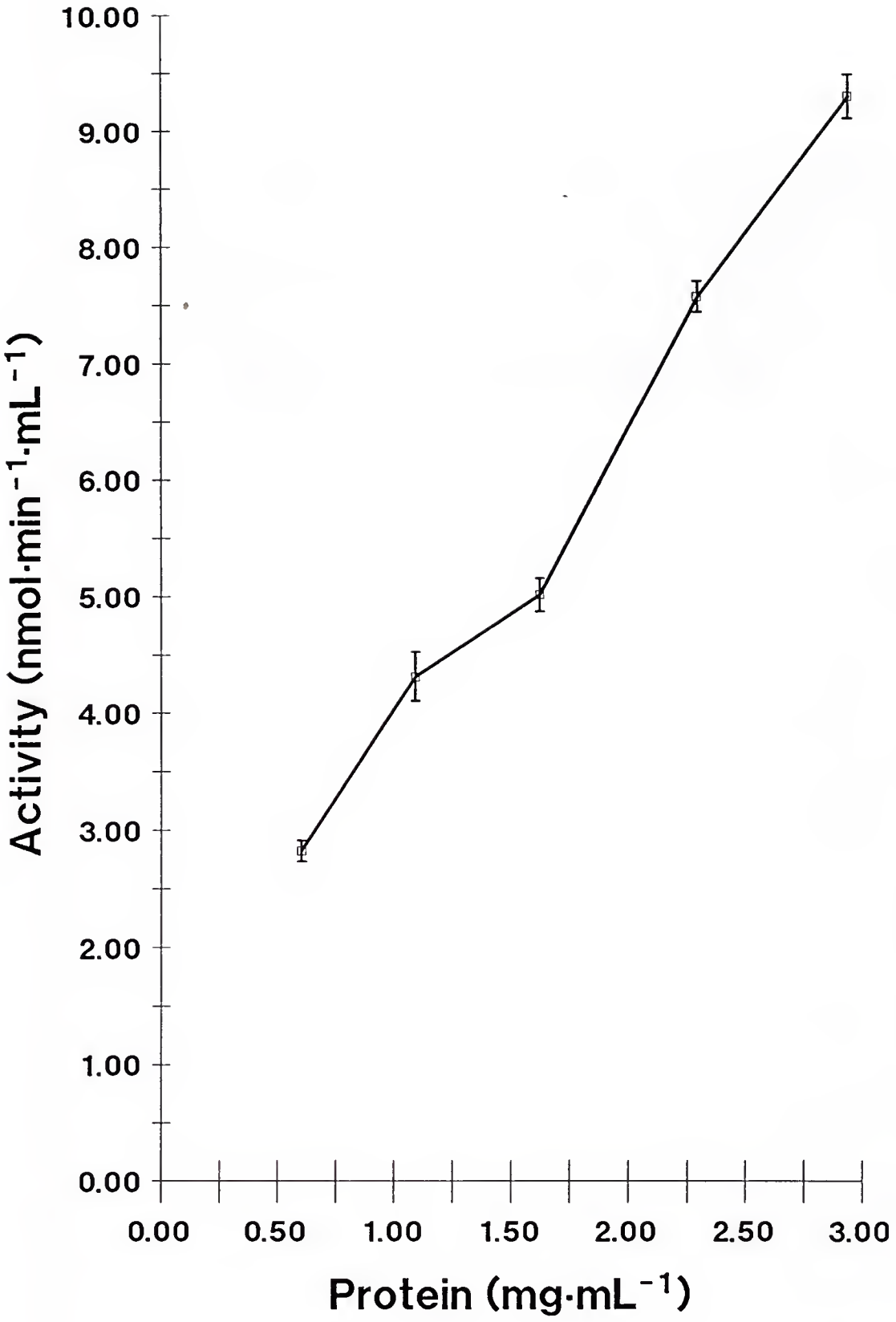

10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Activity (nmol·min⁻¹·mL⁻¹)
0.00
0.50
1.00
1.50
2.00
2.50
3.00
Protein (mg·mL⁻¹)

Figure 24. The effect of varying the concentration of Triton X-100 on Henri-Michaelis-Menten (v versus [S]) curves for α-terpineol dehydratase at 25°C. (Buffer consisted of 10 mM HEPES, pH 7.0. Error bars represent standard deviations, n = 4).

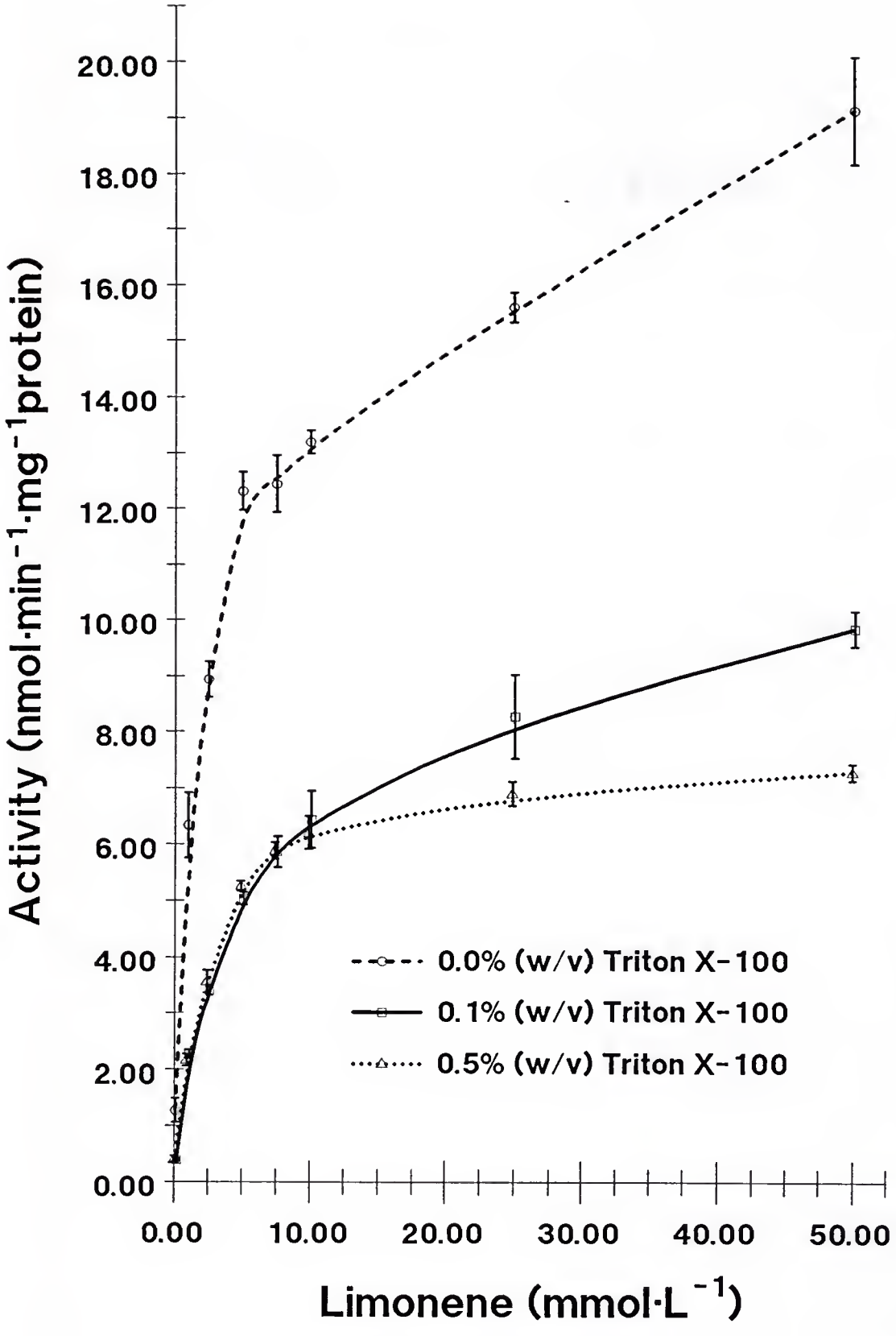

20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Activity (nmol·min⁻¹·mg⁻¹protein)
0.00
10.00
20.00
30.00
40.00
50.00
Limonene (mmol·L⁻¹)
0.0% (w/v) Triton X-100
0.1% (w/v) Triton X-100
0.5% (w/v) Triton X-100

Figure 25. Lineweaver-Burk double reciprocal (1/v versus 1/[S]) plots of the data in Figure 24 for limonene concentrations from 1 mM to 10 mM. (Error bars represent standard deviations, n = 4).

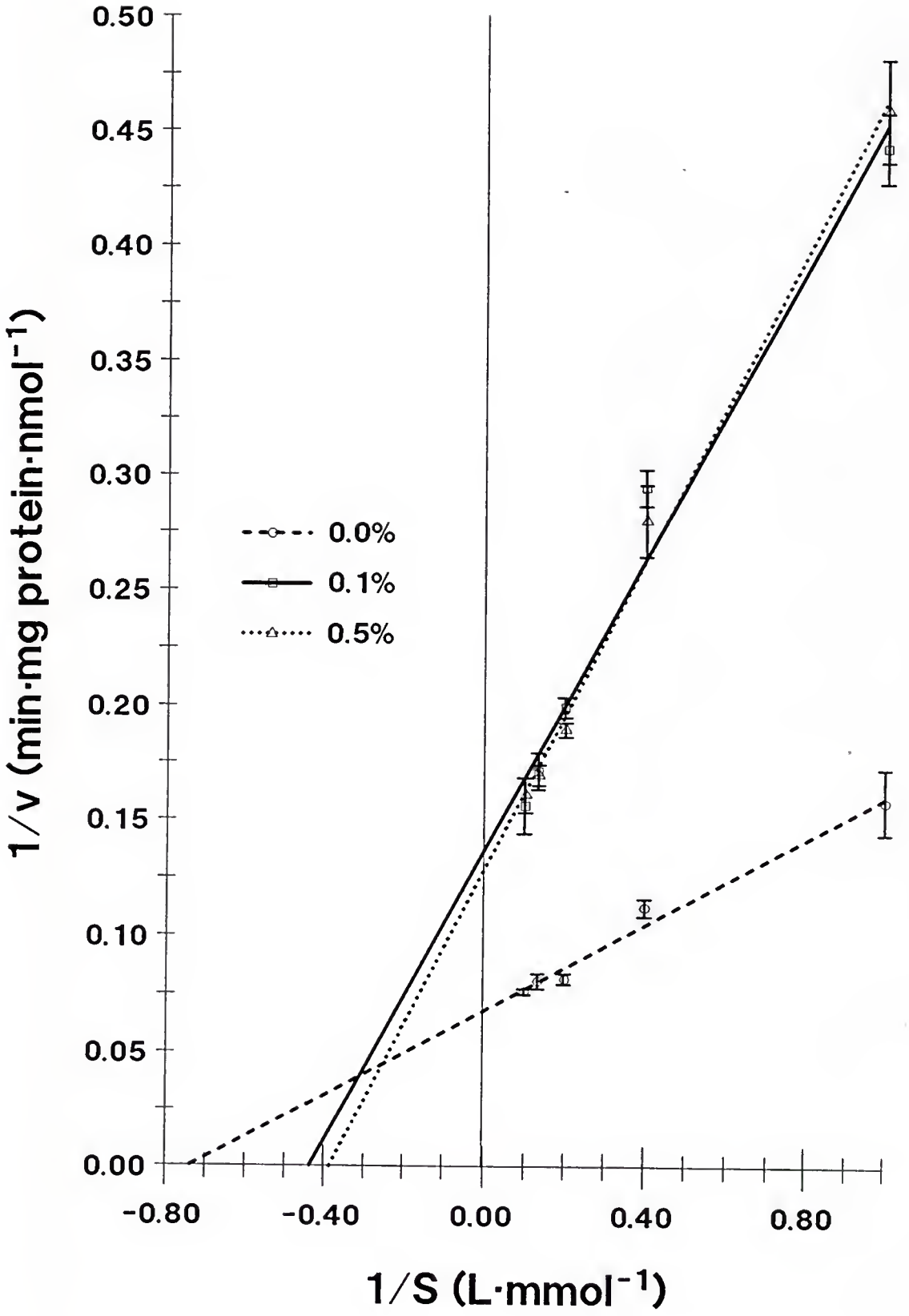

0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1/v (min·mg protein·nmol⁻¹)
0.0%
0.1%
0.5%
-0.80
-0.40
0.00
0.40
0.80
1/S (L·mmol⁻¹)

TABLE 6

Results of linear regression analyses of double reciprocal (1/v versus 1/[S]) Lineweaver-Burk plots obtained at various Triton X-100 concentrations

Triton X-100 Conc. (% w/v)	Linear Regression Output	K_m (mM)	V_{max} (nmol·min^{-1}·mg^{-1} protein)
0.0	$Y^a = 0.091_9(X^b) + 0.067_7$ $X_{SE}{}^c = 0.007_1$, $Y_{SE}{}^d = 0.005_3$ R Squared = 0.997	1.36	14.8
0.1	$Y = 0.315_2(X) + 0.136_7$ $X_{SE} = 0.027_9$, $Y_{SE} = 0.020_8$ R Squared = 0.977	2.31	7.32
0.5	$Y = 0.334_5(X) + 0.129_1$ $X_{SE} = 0.015_0$, $Y_{SE} = 0.011_2$ R Squared = 0.994	2.59	7.75

[a] $Y = 1/v$

[b] $X = 1/[S]$

[c] X_{SE} = standard error of X coefficient

[d] Y_{SE} = standard error of Y constant

and increase in K_m. The degree of inhibition was not proportional to the concentration of detergent in the medium. The value of K_m in the absence of detergent was approximately half the value of K_m in 0.1 or 0.5% detergent. The V_{max} in the absence of detergent was twice as large as the V_{max} in 0.1 or 0.5% detergent. There was no significant difference ($\alpha = 0.05$) between the K_m or V_{max} values determined in 0.1 and 0.5% detergent.

Similar results were observed when studying the effect of Triton X-100 concentration on the catalytic activity of α-terpineol dehydratase (Figure 9). In this study enzyme activity was shown to be insensitive to changes in detergent concentrations between 0.1 and 5.0% (w/v).

This type of inhibition is similar to that observed for some other enzymes after immobilization (Woodward, 1985). The inhibition can be attributed to micro-environmental effects in the vicinity of the enzyme. For example, suppose that each enzyme molecule (or enzyme aggregate) was surrounded by a detergent micelle in which the limonene concentration was lower than in the bulk solution. Consequently, a higher limonene concentration would be required in the bulk solution to saturate the enclosed enzyme, increasing the apparent K_m. The decrease in apparent V_{max} in turn would have resulted from the lower diffusion rate of limonene through the surface of the detergent micelle. The actual system was probably more complicated than described above, since limonene was also

enclosed in detergent micelles. One might consider the possibility of the enzyme and limonene being enclosed in different detergent micelles. Then, the diffusion of available limonene to the enzyme would be even slower. The resulting observed catalytic rate would be less, decreasing the apparent V_{max}.

Replicating K_m in the absence of Triton X-100 was difficult because the partially purified enzyme solutions varied in their composition. Although these solutions contained less than 0.01% (w/v) Triton X-100, slight differences in detergent concentration had a dramatic effect on the results. The most common problem encountered was that the velocity became constant at limonene concentrations higher than 2.5-10 mM, indicating substrate saturation. As discussed earlier, α-terpineol dehydratase in the presence of 0.1% (w/v) Triton X-100 was less sensitive to slight differences in detergent concentration. Average of three replicates of K_m in the presence of 0.1% (w/v) Triton X-100 was 2.18 ± 0.19 mM. Comparison of apparent V_{max} values obtained for different enzyme solutions was not possible because the exact concentration of the enzyme in each solution was unknown.

Stereospecificity and Stereoselectivity

Ideally, the stereoselectivity of α-terpineol dehydratase should be determined by comparing the V_{max}/K_m ratios for both limonene enantiomers. This approach was not practical because pure (4S)-(-)-limonene is not commercially

available. Commercial (4S)-(-)-limonene contained over 10% of the R enantiomer. The stereoselectivity of α-terpineol dehydratase was alternatively determined by incubating the enzyme with racemic limonene and measuring the conversion rate of each limonene enantiomer.

These studies determined that α-terpineol dehydratase catalyzed the hydration of (4R)-(+)-limonene at a faster rate than (4S)-(-)-limonene. In initial studies, enzyme solutions (10 mM HEPES buffer, pH 7.0 containing 0.1% (w/v) Triton X-100) were incubated with 0.1 mM racemic limonene at 25°C for various times and the relative concentration of each limonene enantiomer was determined by chiral GC using a Lipodex C column. Results of these studies (Figures A-7 and A-8) showed that (4R)-(+)-limonene was hydrated at a faster rate than the S-enantiomer. Because of the poor resolution of the limonene enantiomers on this column, the data could not be used to accurately estimate the ratios of the two limonene enantiomers. Furthermore, the fate of each limonene enantiomer could not be determined since α-terpineol enantiomers were not separated.

These problems were overcome by using a Cyclodex B chiral GC column. This column not only achieved better resolution of the limonene enantiomers but also separated α-terpineol enantiomers. A GC profile of the change in concentration of the limonene and α-terpineol enantiomers during the course of the enzymatic hydration of 0.2 mM racemic limonene is shown in Figure 26. Results were

similar to those observed earlier, except enantiomer composition was determined much more accurately. The enzyme stereospecifically converted (4R)-(+)-limonene to (4R)-(+)-α-terpineol and (4S)-(-)-limonene to (4S)-(-)-α-terpineol. These results were expected since Cadwallader et al. (1989) reported that P. gladioli produces pure (4R)-(+)-α-terpineol from (4R)-(+)-limonene.

Plots of relative amounts of each limonene and α-terpineol enantiomer as a function of time (Figure 27) were used to calculate the relative percent concentration of each α-terpineol enantiomer at the indicated times (Table 7). It is apparent from the increase in the relative percent concentration of (4R)-(+)-α-terpineol that the rate of hydration of (4R)-(+)-limonene decreased as a result of its decreasing concentration. In order to obtain a more accurate estimation of the stereoselectivity of α-terpineol dehydratase, initial kinetic rates were used.

Appearance of α-terpineol enantiomers as a function of time at a constant concentration of racemic limonene (50 mM) is shown in Figure 28. The relative percent concentration of (4S)-(-)-α-terpineol at 5 and 10 min was 9.2 and 10.2 respectively. At 2 min the relative percent concentration was 13.7. From the curvature of both plots it appeared that the concentration of the S-enantiomer was over-estimated. Replication of the experiment using a different enzyme solution gave a relative percent concentration of 10.3 for (4S)-(-)-α-terpineol at 2 min. It was concluded from these

results that α-terpineol dehydratase stereoselectively catalyzed the hydration of (4R)-(+)-limonene approximately ten times faster than (4S)-(-)-limonene (Figure 29).

Figure 26. GC profile of the change in concentration of limonene and α-terpineol enantiomers during the course of the hydration of 0.2 mM racemic limonene by α-terpineol dehydratase at 25°C. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. GC conditions are given in Materials and Methods.)

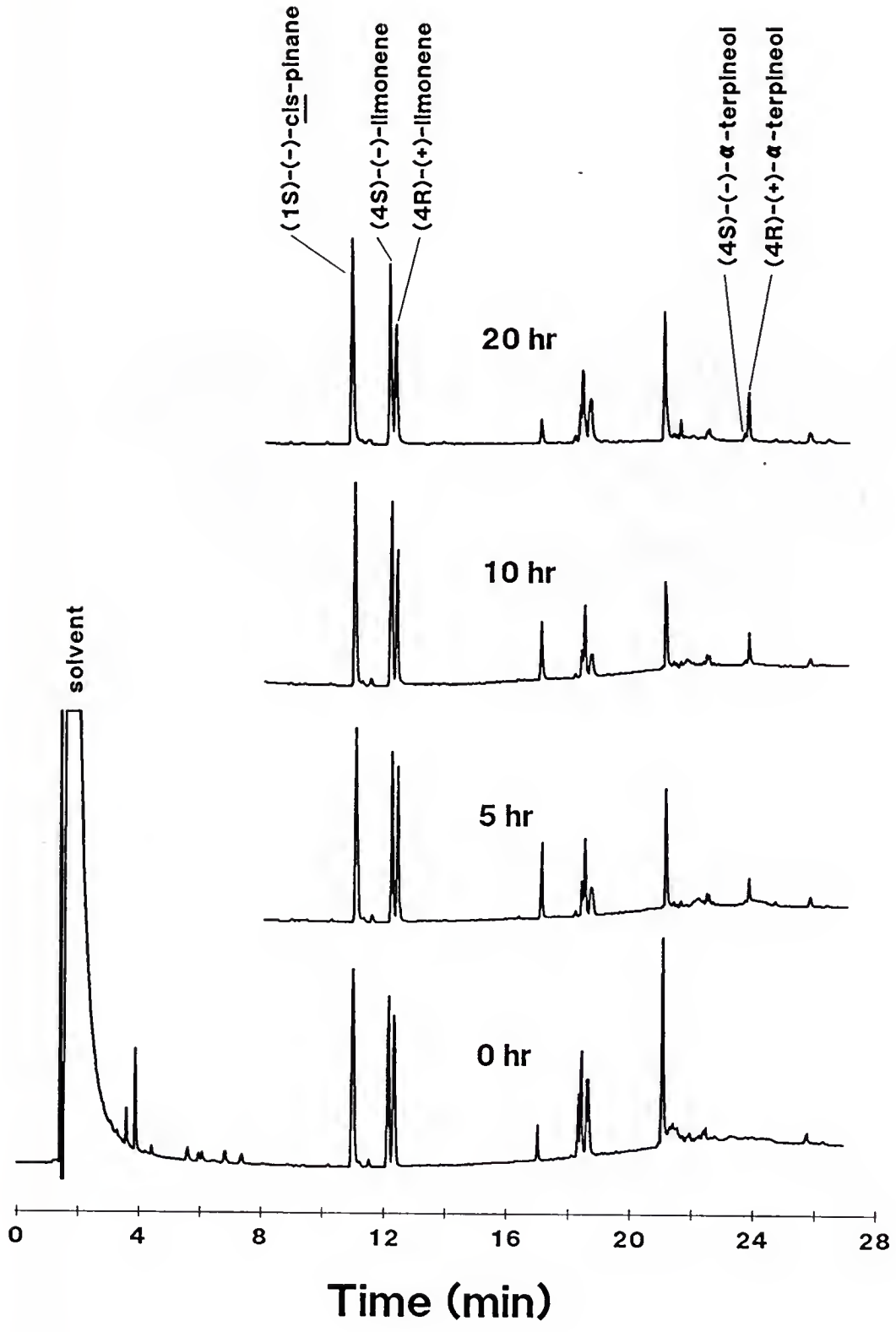

(1S)-(-)-cis-pinane
(4S)-(-)-limonene
(4R)-(+)-limonene
(4S)-(-)-α-terpineol
(4R)-(+)-α-terpineol
20 hr
10 hr
5 hr
0 hr
solvent
0
4
8
12
16
20
24
28
Time (min)

Figure 27. Change in concentration of limonene and α-terpineol enantiomers during the course of the hydration of 0.2 mM racemic limonene by α-terpineol dehydratase at 25°C. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)

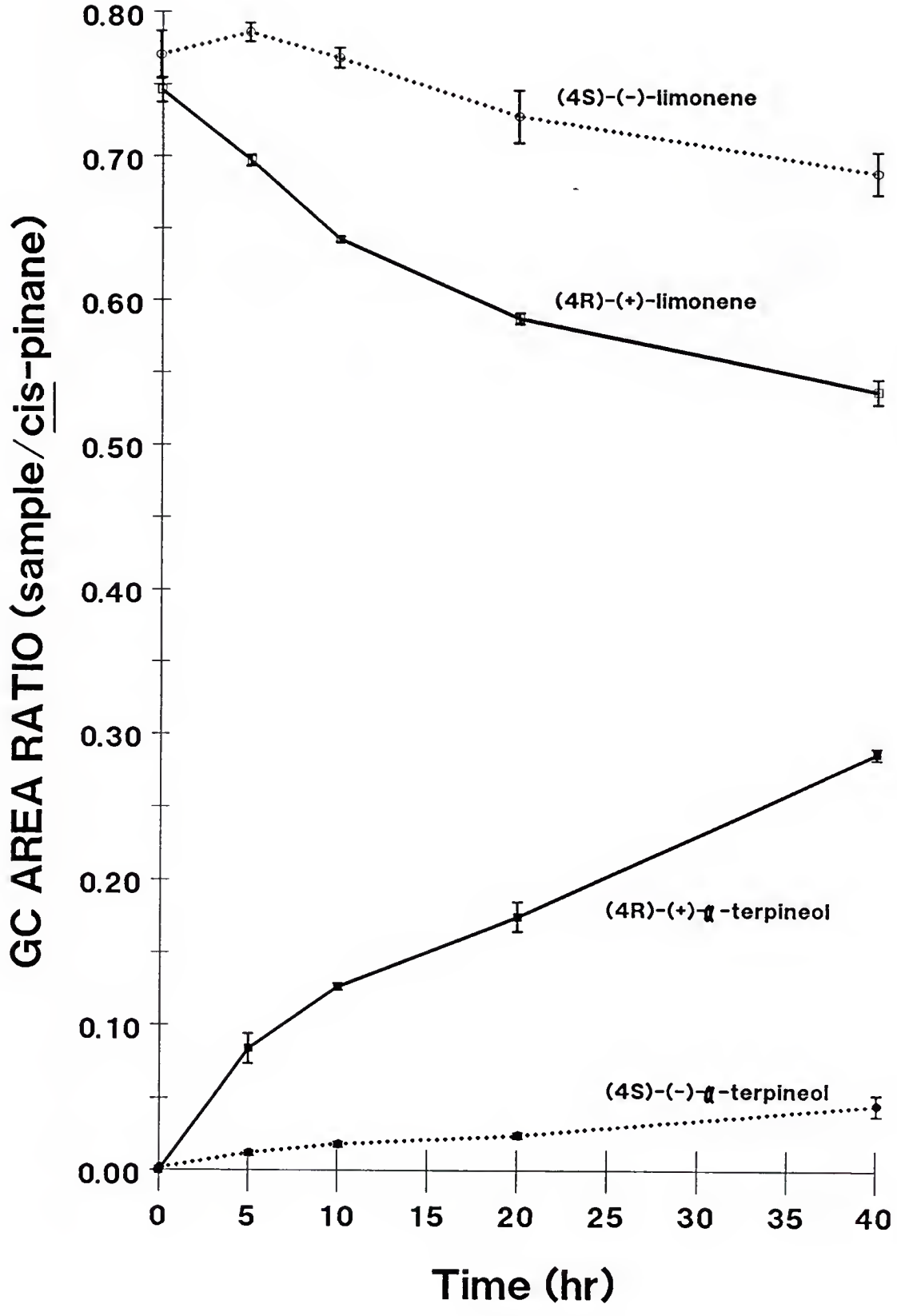
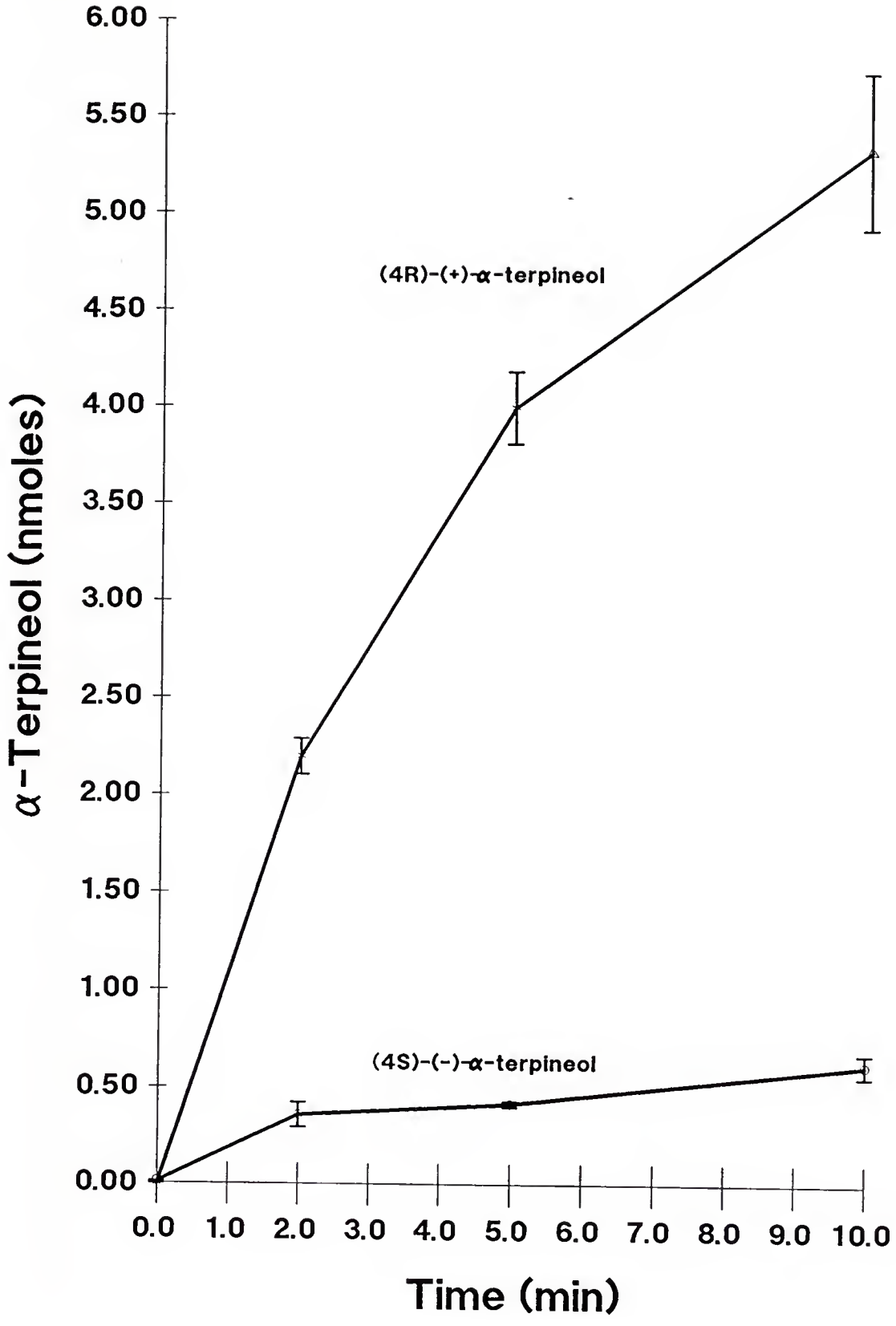

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
GC AREA RATIO (sample/cis-pinane)
(4S)-(-)-limonene
(4R)-(+)-limonene
(4R)-(+)-α-terpineol
(4S)-(-)-α-terpineol
0
5
10
15
20
25
30
35
40
Time (hr)

TABLE 7

Relative percent concentration of α-terpineol enantiomers as a function of time for the hydration of 0.2 mM racemic limonene by α-terpineol dehydratase at 25°C

	Relative Percent Concentration	
Time (hr)	(4S)-(-)-α-Terpineol	(4R)-(+)-Terpineol
0	0.0	0.0
5	10.8	89.2
10	11.3	88.7
20	11.4	88.6
40	13.3	86.7

Figure 28. Formation of α-terpineol enantiomers during the course of the hydration of 50 mM racemic limonene by α-terpineol dehydratase at 25°C. (Buffer consisted of 10 mM HEPES, pH 7.0 and 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 4.)


6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
α-Terpineol (nmoles)
(4R)-(+)-α-terpineol
(4S)-(-)-α-terpineol
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Time (min)

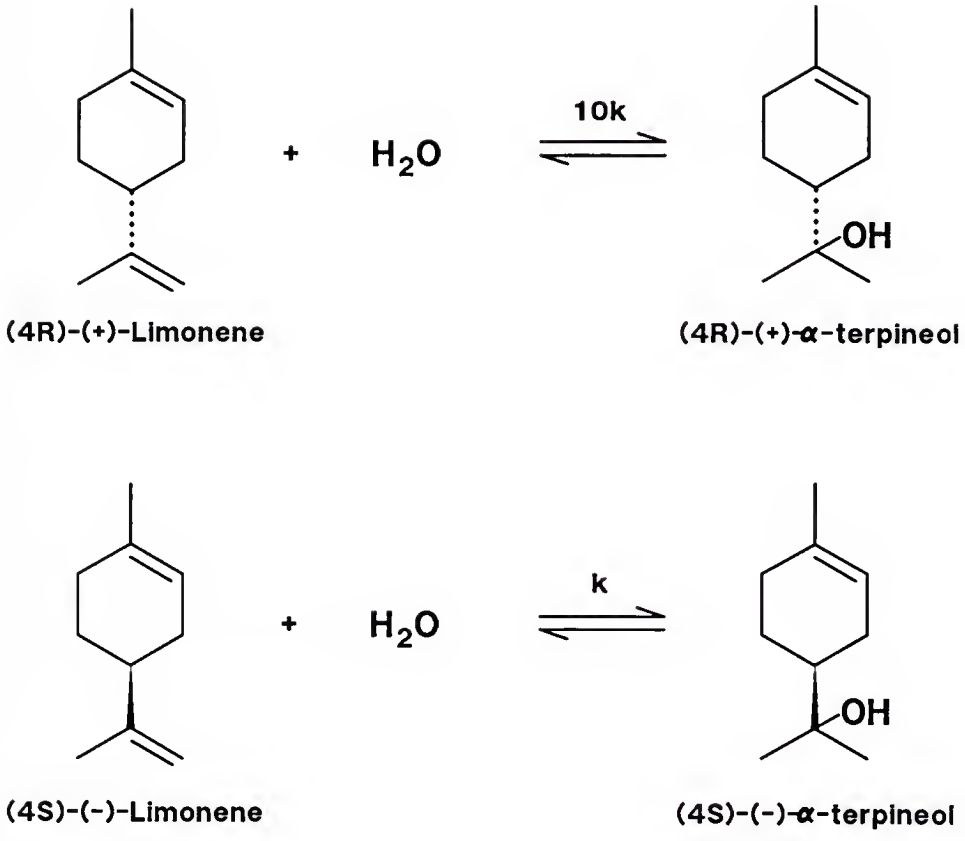


Figure 29. Stereo-specificity and -selectivity of α-terpineol dehydratase. (Note that k represents the rate constant for the hydration of (4S)-(-)-limonene.)

CONCLUSIONS

In this study, the enzyme-catalyzed hydration of limonene to α-terpineol was investigated using α-terpineol dehydratase from P. gladioli. After solubilization and partial purification of the enzyme, several of its physical and kinetic properties were characterized. The following conclusions were drawn from the results of this study:

1) After disruption of P. gladioli cells, α-terpineol dehydratase remains associated with heterogeneous insoluble particulate material.
2) α-Terpineol dehydratase can be effectively solubilized from the particulate material by extraction with 10 mM HEPES buffer, pH 7.0 containing 2.0% (w/v) Triton X-100 and 0.5 M sodium trichloroacetate.
3) α-Terpineol dehydratase is a hydrophobic protein since inclusion of 1.0% (w/v) Triton X-100 in the gel filtration buffer is required for maximum recovery of enzyme activity.
4) Two soluble forms exist in 1.0% (w/v) Triton X-100: presumably a native enzyme monomer and a dimer with apparent molecular weights of 94,500 and 206,500 daltons, respectively. SDS-PAGE analysis of enzyme fractions revealed that a 92,000 dalton polypeptide was

enriched by partial purification of the enzyme, suggesting that at least some portion of the α-terpineol dehydratase monomer was not completely fractionated into smaller subunits in SDS denaturing conditions.

5) The reaction, limonene $\xrightarrow{\text{enzyme}}$ α-terpineol, is not readily reversible, since the enzyme-catalyzed dehydration of α-terpineol to limonene could not be demonstrated even in media with reduced water concentration.

6) α-Terpineol dehydratase activity is low in non-aqueous solvents due to denaturation and conformational changes, and not because of unfavorable equilibrium conditions. A slightly acidic environment increased α-terpineol dehydratase activity, and at the same time decreased its stability. It was concluded that the activity decrease at low pH was due to denaturation instead of improper enzyme ionization.

7) α-Terpineol dehydratase is heat labile, possibly reflecting its high molecular weight. The activity of α-terpineol dehydratase is insensitive to changes in temperature as indicated by the low E_a (21.6 ± 2.9 $kJ \cdot mol^{-1}$) and Q_{10} (1.37 ± 0.07) values.

8) Triton X-100 inhibits α-terpineol dehydratase activity, causing an increase in the apparent K_m and decrease in apparent V_{max}. The apparent K_m of α-terpineol dehydratase in 0.1% (w/v) Triton X-100 was 2.18 ± 0.19 mM. The numerical value of K_m is important for design of an enzymatic conversion process, since an enzyme is most efficient at substrate concentrations near K_m.

10) α-Terpineol dehydratase stereospecifically catalyzes the hydration of (4R)-(+)-limonene to (4R)-(+)-α-terpineol and the hydration of (4S)-(-)-limonene to (4S)-(-)-α-terpineol. The enzyme is stereoselective for (4R)-(+)-limonene, with the rate for hydration of (4R)-(+)-limonene being approximately ten times faster than the rate for the hydration of (4S)-(-)-limonene.

In recent years there has been an increased demand for natural flavor and aromas. Use of a natural process for the production of (4R)-(+)-α-terpineol from (4R)-(+)-limonene has economic potential because the annual consumption of α-terpineol is high; while at the same time it has greater commercial value than limonene. Commercial value of pure (4R)-(+)-α-terpineol is unknown because it is not available. An additional use of α-terpineol dehydratase could be for the resolution of commercial racemic limonene (dipentene) into pure (4S)-(-)-limonene; while at the same time producing 90% pure (4R)-(+)-α-terpineol.

APPENDIX

Figure A-1. Standard curve for α-terpineol using 0.5 ppm n-decanol as internal standard. (Error bars represent standard deviations, n = 4.)

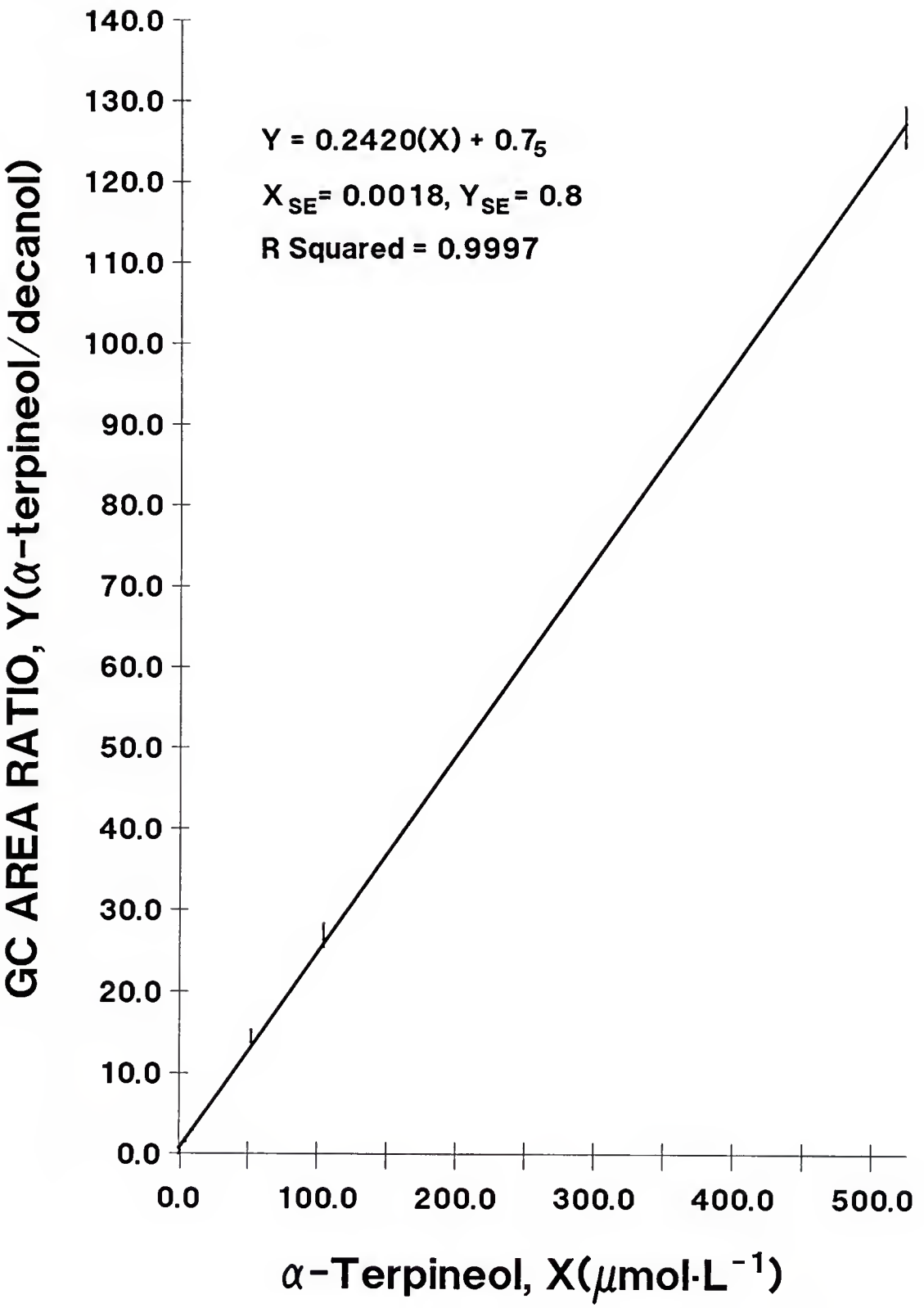

Y = 0.2420(X) + 0.7₅
X SE= 0.0018, Y SE = 0.8
R Squared = 0.9997
GC AREA RATIO, Y(α-terpineol/decanol)
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
100.0
200.0
300.0
400.0
500.0
α-Terpineol, X(μmol·L⁻¹)

Figure A-2. Gas chromatogram of a typical enzyme assay extract using a 0.53 mm i.d. x 15 m SE 54 fused silica capillary column.

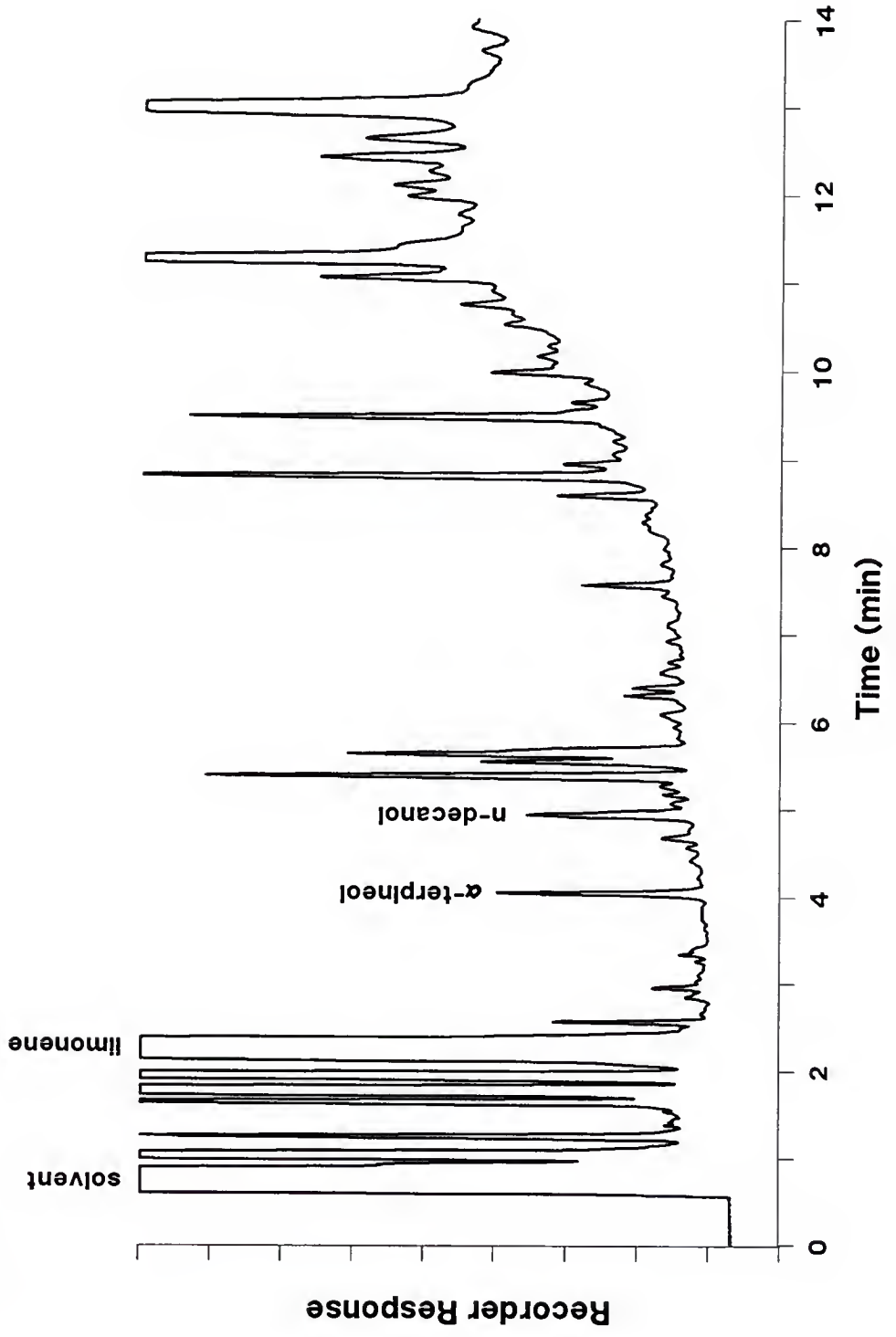

Recorder Response
Time (min)
0
2
4
6
8
10
12
14
solvent
limonene
α-terpineol
n-decanol

Figure A-3. Absorbance (275 nm) versus Triton X-100 concentration for standard solutions and their respective UF permeates. (Note that the regression line for the UF permeate excludes the data for 0.1% (w/v) Triton X-100. Error bars represent standard deviations, n = 3.)

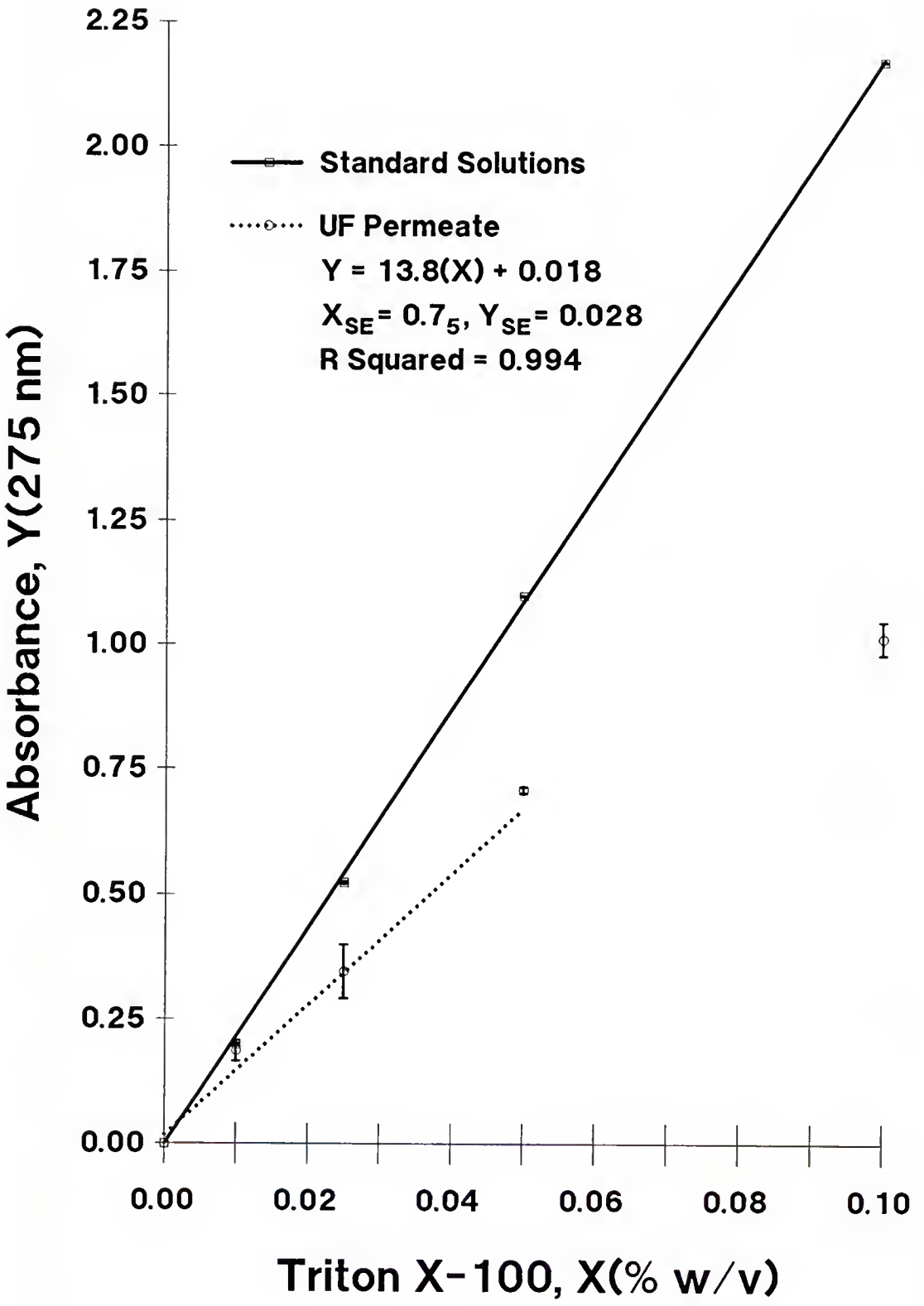

2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance, Y(275 nm)
Standard Solutions
UF Permeate
Y = 13.8(X) + 0.018
X_{SE}= 0.7$_5$, Y_{SE}= 0.028
R Squared = 0.994
0.00
0.02
0.04
0.06
0.08
0.10
Triton X-100, X(% w/v)

Figure A-4. Chiral gas chromatogram of 1 μL enzyme assay extract (from stereospecificity/ stereoselectivity initial rate study) using a 0.254 mm i.d. x 30 m Cyclodex B fused silica capillary column.

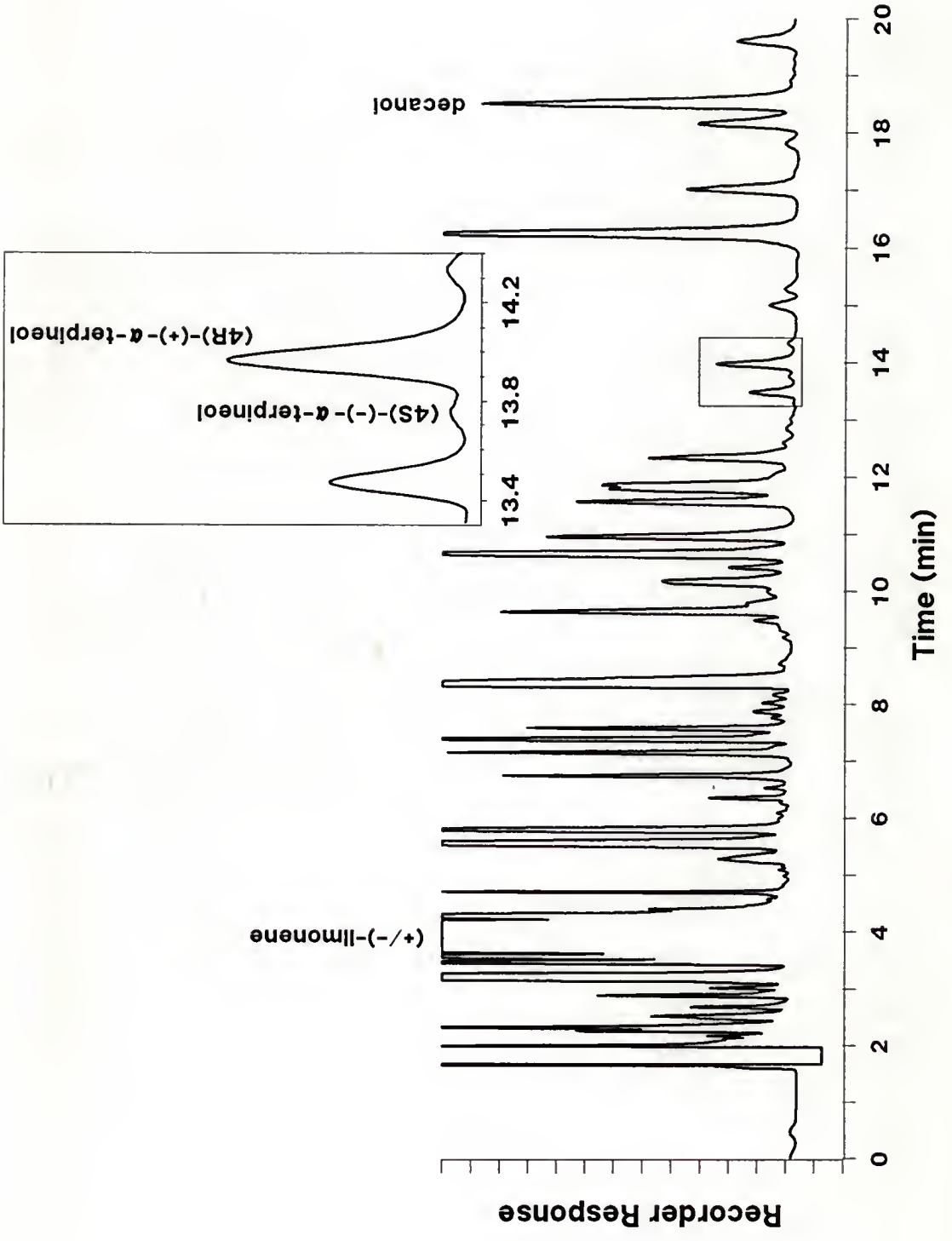

Recorder Response
Time (min)
0
2
4
6
8
10
12
14
16
18
20
(+/-)-limonene
decanol
13.4
13.8
14.2
(4S)-(-)-α-terpineol
(4R)-(+)-α-terpineol

Figure A-5. Standard curves for (4R)-(+)-α-terpineol and (4S)-(-)-α-terpineol using 5 ppm n-decanol as internal standard. (Error bars represent standard deviations, n = 4.)

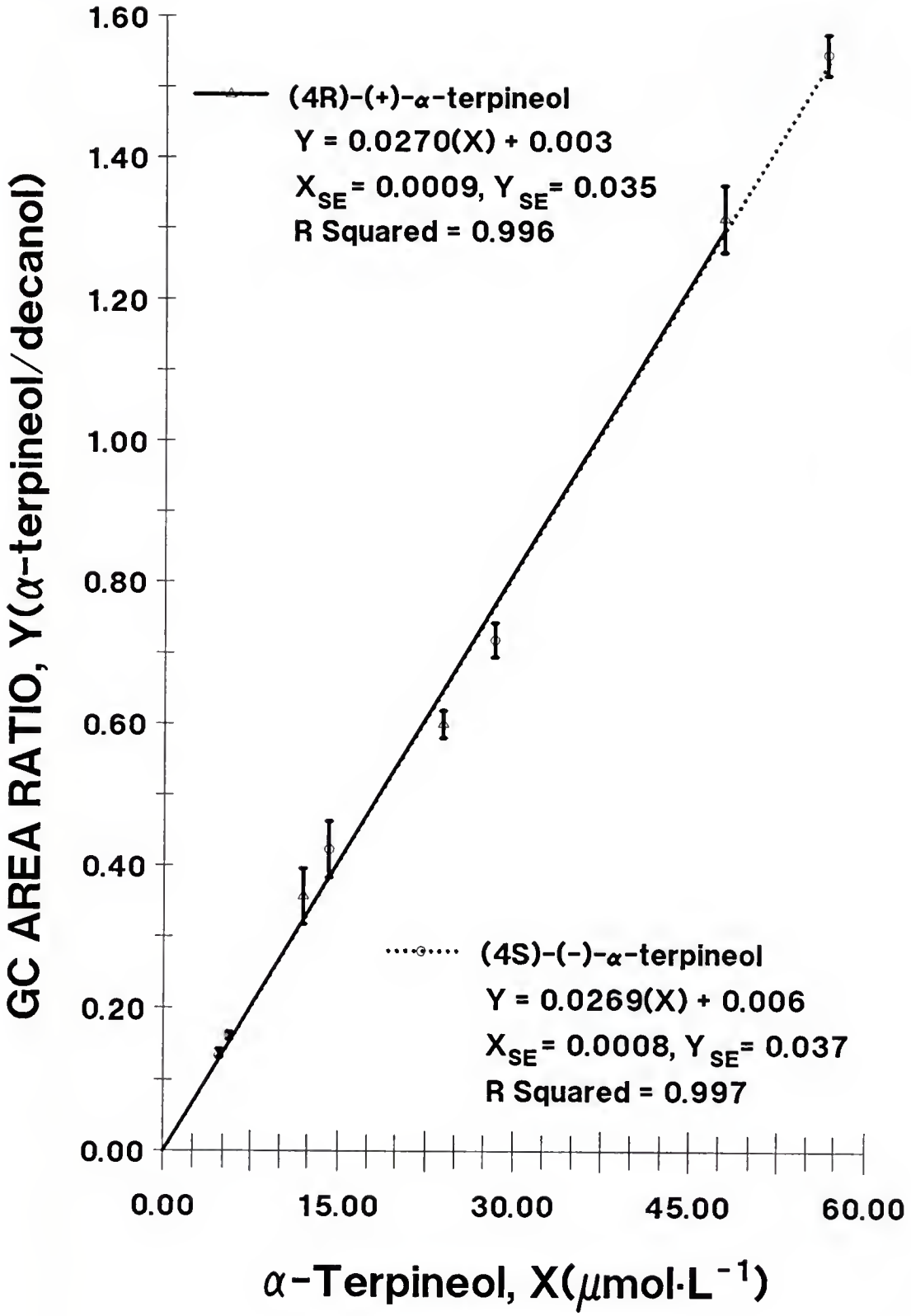

(4R)-(+)-α-terpineol
Y = 0.0270(X) + 0.003
X_SE = 0.0009, Y_SE = 0.035
R Squared = 0.996
(4S)-(-)-α-terpineol
Y = 0.0269(X) + 0.006
X_SE = 0.0008, Y_SE = 0.037
R Squared = 0.997
GC AREA RATIO, Y(α-terpineol/decanol)
α-Terpineol, X(μmol·L⁻¹)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
15.00
30.00
45.00
60.00

Figure A-6. Growth curves of P. gladioli at 30°C in liquid mineral medium containing 0.2% (v/v) limonene at various pH values.

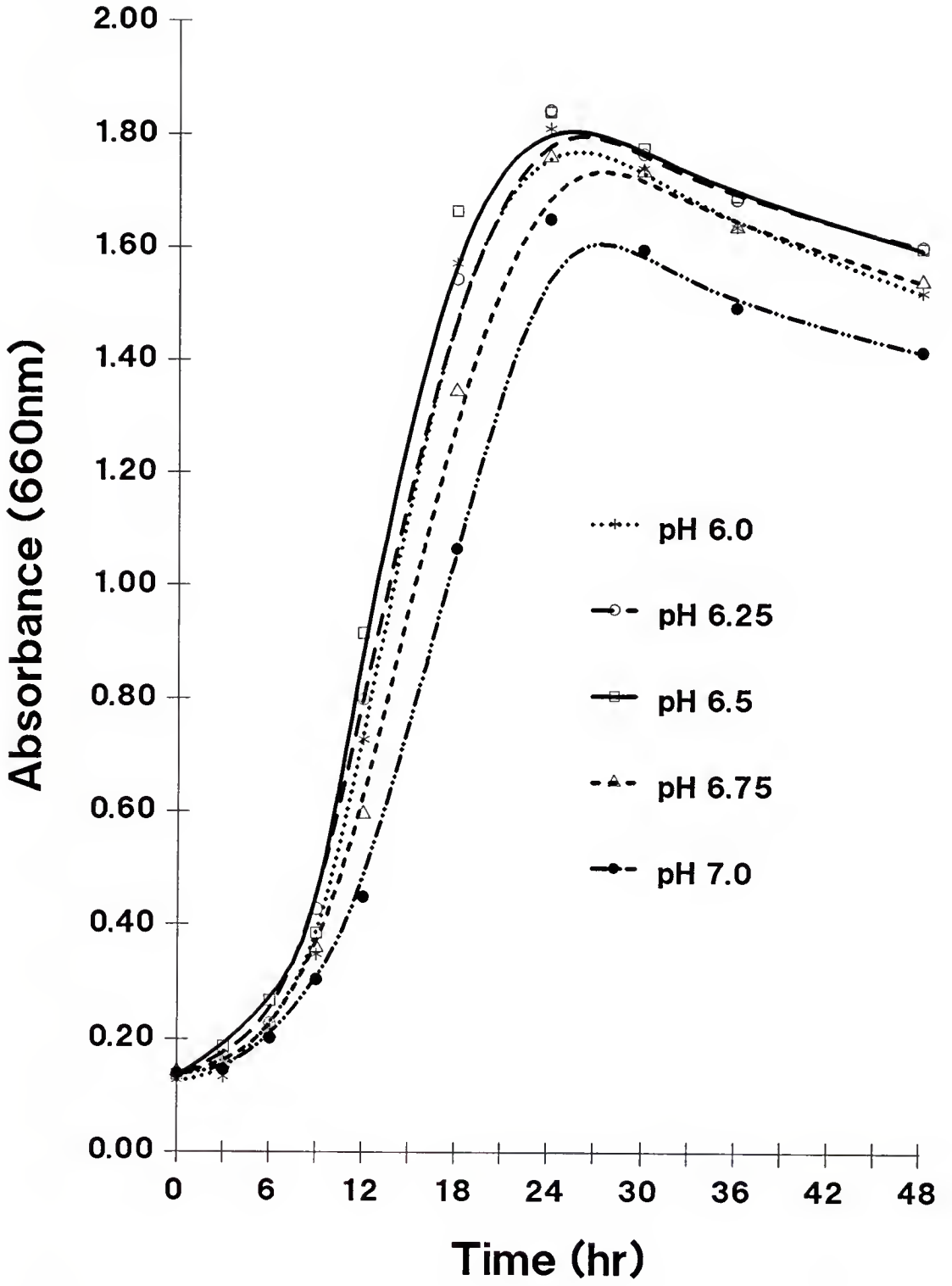

2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Absorbance (660nm)
0
6
12
18
24
30
36
42
48
Time (hr)
pH 6.0
pH 6.25
pH 6.5
pH 6.75
pH 7.0

Figure A-7. GC profile of change in concentration of limonene enantiomers during the course of the hydration of 0.1 mM racemic limonene by α-terpineol dehydratase at 25°C. [Buffer consisted of 10 mM HEPES, pH 7.0 containing, 0.1% (w/v) Triton X-100. Separations were performed on a 0.25 mm i.d. x 50 m Lipodex C glass capillary column with a film thickness of 0.1 μm (Macherey-Nagel, Düren, Germany). GC conditions were as follows: 1 μL injection with 1:100 split; helium carrier gas, 0.74 $mL \cdot min^{-1}$; injector port at 200°C; detector at 250°C; column temperature isothermal at 60°C.]

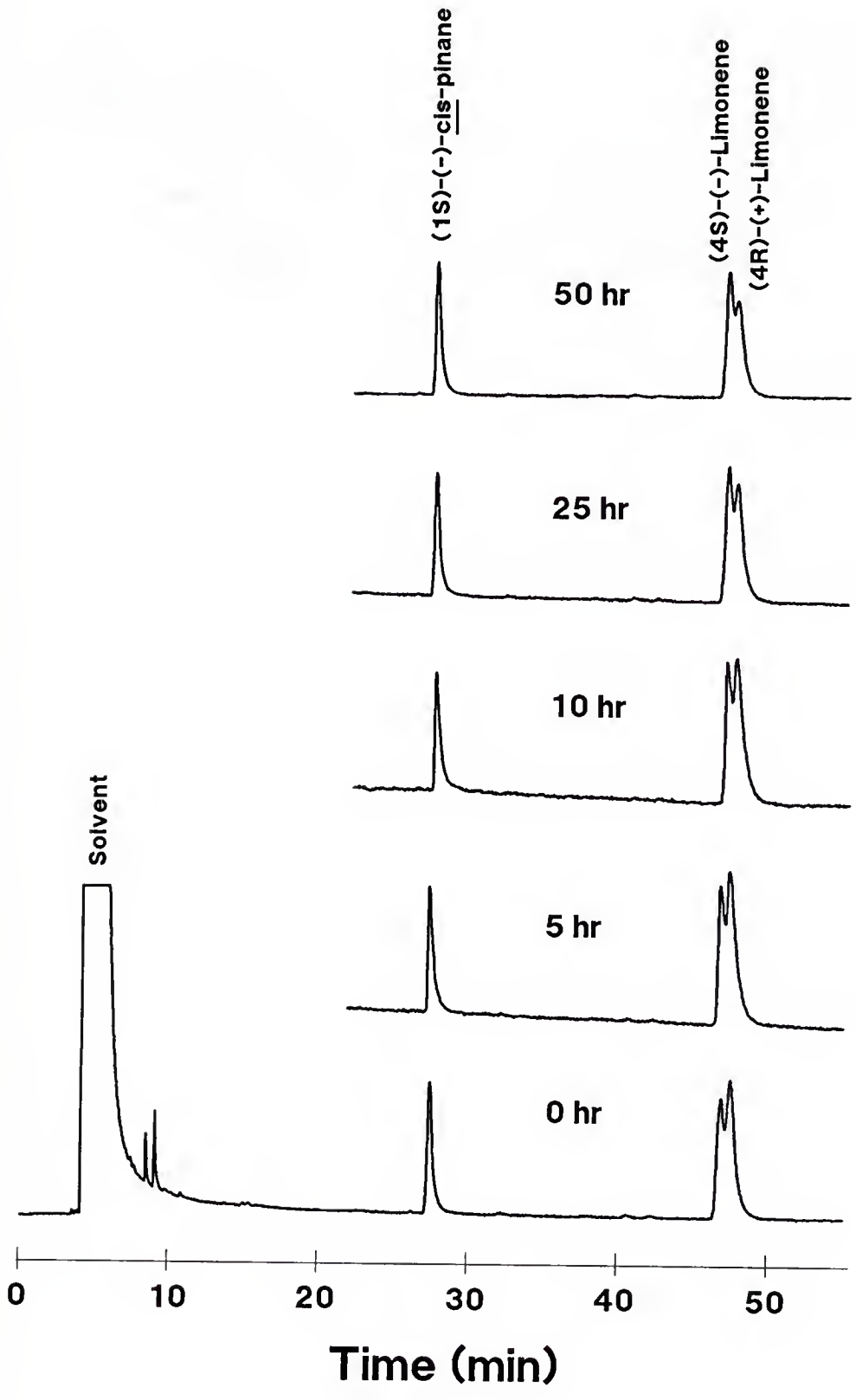
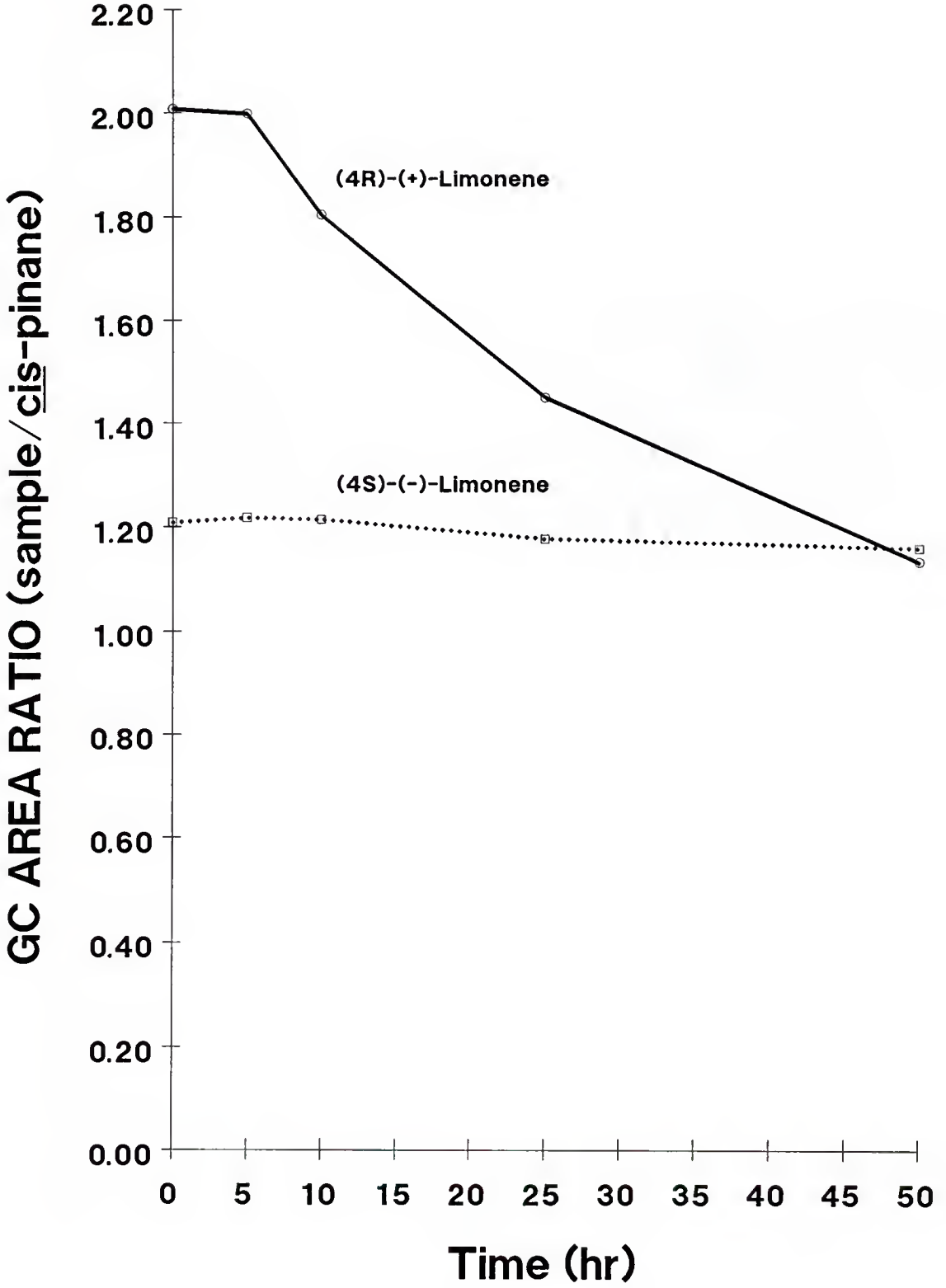

(1S)-(-)-cis-pinane
(4S)-(-)-Limonene
(4R)-(+)-Limonene
50 hr
25 hr
10 hr
Solvent
5 hr
0 hr
0
10
20
30
40
50
Time (min)

Figure A-8. Disappearance of limonene enantiomers during the course of the hydration of 0.1 mM racemic limonene by α-terpineol dehydratase at 25°C.


GC AREA RATIO (sample/cis-pinane)
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
(4R)-(+)-Limonene
(4S)-(-)-Limonene
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

REFERENCES

Abraham, W.R., Hoffmann, H.M.R., Kieslich, K., Reng, G., and Stumpf, B. 1985. Microbial transformation of some monoterpenoids and sesquiterpenoids. In "CIBA Foundation Symposium III. Enzymes in Organic Synthesis." Pitman, London. pp. 146-157.

Abraham, W.R., Kieslich, K., Reng, G., and Stumpf, B. 1984. Formation and preparation of 1,2-trans-glycols from various monoterpenes with 1-menthene skeletons by microbial transformations with Diplodia gossypina. Third Eur. Congr. Biotechnol. Vol 1. Munich, Sept. 10. Verlag Chemie, Deerfield Beach, FL. pp. 245-248.

Alder-Nissen, J. 1985. "Enzymatic Hydrolysis of Food Proteins." Elsevier, London.

American Public Health Association. 1989. "Standard Methods for the Examination of Water and Wastewater," 17th ed. Clesceri, L.S., Greenberg, A.E., and Trussel R.R. (Eds.). American Public Health Association, Washington, DC.

Anon. 1989. Total production of d-limonene. In "Statistical Summary 1988-89 Season." Florida Citrus Processors Association, Winter Haven, FL. p. 1D.

Anon. 1990. Chemical prices. In "Chemical Marketing Reporter," Vol. 238 (12). Van, H., Deyrup, C.A., and McCoy, M. (Eds.). Schnell Publishing Co., Ltd., New York. pp. 38,40,44.

Arctander, S. 1969a. Dipentene: 1074. In "Perfume and Flavor Chemicals (Aroma Chemicals)," Vol. 1. Steffen Arctander, Montclair, NJ.

Arctander, S. 1969b. d-Limonene: 1800. In "Perfume and Flavor Chemicals (Aroma Chemicals)," Vol. 2. Steffen Arctander, Montclair, NJ.

Arctander, S. 1969c. l-Limonene: 1801. In "Perfume and Flavor Chemicals (Aroma Chemicals)," Vol. 2. Steffen Arctander, Montclair, NJ.

Arctander, S. 1969d. α-Terpineol: 2877. In "Perfume and Flavor Chemicals (Aroma Chemicals)," Vol. 2. Steffen Arctander, Montclair, NJ.

Armstrong, D.W. and Yamazaki, H. 1986. Natural flavours production: a biotechnological approach. Trends in Biotechnol. 4: 264-268.

Ballal, N.R., Bhattacharyya, P.K., and Rangachari, P.N. 1966. Perillyl alcohol dehydrogenase from a soil pseudomonad. Biochem. Biophys. Res. Commun. 23: 473-478.

Ballal, N.R., Bhattacharyya, P.K., and Rangachari, P.N. 1967. Perillyl aldehyde dehydrogenase from a soil pseudomonad. Biochem. Biophys. Res. Commun. 23: 473-478.

Bowen, E.R. 1975. Potential by-products from the microbial transformation of d-limonene. Proc. Fla. State Hort. Soc. 88: 305-308.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principles of protein dye-binding. Anal. Biochem. 72: 248-254.

Bradshaw, W.H., Conrad, H.E., Corey, E.J., Gunsalus, I.C., and Lednicer, D. 1959. Microbiological degradation of (+)-camphor. J. Am. Chem. Soc. 81: 5507.

Brink, L.E.S., Tramper, J., Luyben, K.C.A.M., Riet, K. Van't. 1988. Biocatalysis in organic media. Enzyme and Microbiol. Technol. 10: 736-743.

Brown, H.C., Geoghegan, P.J., Jr., Lynch, G.J., and Kurek, J.T. 1972. Solvomercuration-demercuration. IV. The monohydration of representative dienes via oxymercuration-demercuration. J. Org. Chem. 37: 1941-1947.

Buchel, J. 1989. Flavoring with citrus oils. Perfumer & Flavorist 14: 22-26.

Buckholz, L.L., Jr. and Daun, H. 1978. Instrumental and sensory characterization of orange oil oxidation. J. Food. Sci. 43: 535-539.

Cadwallader, K.R., Braddock, R.J., Parish, M.E., and Higgins, D.P. 1989. Bioconversion of (+)-limonene by Pseudomonas gladioli. J. Food Sci. 54: 1241-1245.

Cambou, B. and Klibanov, A.M. 1984. Preparative production of optically active esters and alochols using esterase-catalyzed stereospecific transesterification in organic media. J. Am. Chem. Soc. 106: 2687-2692.

CRC. 1986. "CRC Handbook of Chemistry and Physics," 67th ed. CRC Press, Inc., Boca Raton, FL.

Dhavlikar, R.S. and Bhattacharyya, P.K. 1966. Microbiological transformations of terpenes: Part VIII. Fermentation of limonene by a soil pseudomonad. Indian J. Biochem. 3: 144-157.

Dhavlikar, R.S., Rangachari, P.N., and Bhattacharyya, P.K. 1966. Microbiological transformations of terpenes: Part IX. Pathways of degradation of limonene in a soil pseudomonad. Indian J. Biochem. 3: 158-164.

Dhere, S.G. and Dhavlikar, R.S. 1970. Microbial transformations of terpenoids: limonene. Sci. Cult. 36: 292.

Doerner, K.C. and White, B.A. 1990. Detection of glyco-proteins separated by nondenaturing polyacrylamide gel electrophoresis using the periodic acid-Schiff stain. Anal. Biochem. 187: 147-150.

Eiberger, L.L. and Wasserman B.P. 1987. Partial purification of digitonin-solubilized ß-glucan synthase from red beet root. Plant Physiol. 83: 982-967.

Engel, K.-H., Bohnen, M., and Tressl, R. 1989. Kinetic resolution of heptan-2-ol-enantiomers by lipase-catalysed esterification in organic solvent. Z. Lebensm. Unters. Forsh. 188: 144-147.

FMC. 1976. d-Limonene. Brochure No. 460SA225576. FMC Corporation, Lakeland, FL.

Gerlach, D., Schneider, S. Göllner, T., Klim. K.S., and Schreier P. 1987. Screening of lipases for enantiomer resolution o secondary alcohols by esterification in organic medium. In "Bioflavor'87." Schreier, P. (Ed.). Walter de Gruyter & Co., New York. pp. 543-554.

Hammes, G.G. 1978. "Principles of Chemical Kinetics." Academic Press, New York.

Hatefi, Y. and Hanstein, W.G. 1974. Destabilization of membranes with chaotropic ions. Meth. Enzymol. 31: 770-790.

International Union of Biochemistry. 1984. "Enzyme Nomenclature 1984." Webb, E.C. (ED.) Academic Press, Inc., Orlando, FL.

Karp, F., Milhaliak, D.A., Harris, J., and Croteau, R. 1990. Monoterpene biosynthesis: specificity of the hydroxylations of (-)-limonene by enzyme preparations from (Mentha piperita), spearmint (Mentha spicata), and (Perilla frutescens) leaves. Arch. of Biochem. and Biophys. 276: 219-226.

Kesterson, J.W. and Braddock, R.J. 1976. By-products and specialty products of Florida citrus. Fla. Agr. Exp. Sta. Bull. 784: 33-34.

Khmelnitsky, Y.L., Levashov, A.V., Klyachko, N.L., and Martinek, K. 1988. Engineering biocatalytic systems in organic media with low water content. Enzyme Microbiol. Technol. 10: 710-724.

Kisee, H. Fujimoto, K., and Noritomi, H. 1988. Enzymatic reactions in aqueous-organic media. VI. Peptide synthesis by α-chymotrypsin in hydrophilic organic solvents. J. Biotechnol. 8: 279-290.

Klibanov, A.M. 1986. Enzymes that work in organic solvents. CHEMTECH 16: 354-359.

Knorr, D., Beaumont, M.D., Caster, C.S., Dörnenburg, H., Gross, B., Pandya, Y., and Romagnoli, L.G. 1990. Plant tissue culture for the production of naturally derived food ingredients. Food Technol. 44(6): 71-79.

Kraidman, G., Mukherjee, B.B., and Hill, I.D. 1969. Conversion of d-limonene into an optically active isomer of α-terpineol by a Cladosporium species. Bacteriol. Proc. 69: 63.

Legoy, M.D., Klim, H.S., and Thomas, D. 1985. Use of alcohol dehydrogenase for flavour aldehyde production. Process Biochem. 20: 145-148.

Madhyastha, K.M., Rangachari, P.N., Rangachari, R.M., and Bhattacharyya, P.K. 1968. Microbiological transformation of terpenes. XV. Enzyme systems in the catabolism of p-cymene in PL-strain. Indian J. Biochem. 5:161.

Massaldi, H.A. and King, C.J. 1973. Simple technique to determine solubilities of sparingly soluble organics: solubility and activity coefficients of d-limonene, n-butylbenzene, and n-hexyl acetate in water and sucrose solutions. J. Chem. Eng. Data. 18: 393-397.

Matsubara, Y., Tanaka, K., Urata, M., Fukunaga, T., Kuwata, M., and Takahashi, K. 1975. Hydration of terpenes. VI. Novel synthesis of optically active α-terpineol and 2,6-dimethyl-7-octen-2-ol. Nippon Kagaku Kaishi. 5:855-858.

Mattson, R.H. 1984. Turpentine, a by-product of the kraft paper industry. Part 1. Naval Stores Rev. 46(5): 10-16.

Monod J. and Wollman E. 1947. L'inhibition de la croissance de l'adaption enzymatique chez les bacteries par le bacteriophage. Ann. Inst. Pasteur 73: 937-956.

Murdock, D.I. and Hunter, G.L.K. 1970. Bacteriological contamination of some citrus oils during processing. J. Food Sci. 35: 652-655.

Murdock, D.I., Hunter, G.L.K., Bucek, W.A., and Brent, J.A. 1967. Relationship of bacterial contamination in orange oil recovery system to quality of finished product. Proc. Fla. State Hort. Soc. 80: 267-280.

Murdock, D.I., Hunter, G.L.K., Bucek, W.A., and Brent, J.A. 1969. Relationship of bacterial contamination in orange oil recovery system to quality of finished product. Food Technol. 23(5): 97-100.

Mukherjee, B.B., Kraidman, G., and Hill, I.D. 1973. Synthesis of glycols by microbial transformation of some monocyclic terpenes. Appl. Microbiol. 25: 447-453.

Nakamura, K., Aizawa, M., and Miyawaki, O. 1988. "Biotechnology Monographs. Electro-enzymology, Coenzyme Regeneration," Vol. 4. Springer-Verlag, New York.

Nalecz, K.A., Bolli, R., and Azzi, A. 1986. Molecular weight estimation of membrane proteins: gel permeation chromatography and sucrose gradient centrifugation. In "Membrane Proteins: Isolation and Characterization." Azzi, A.A., Masotti, L., and Vecli, A. (Ed.). Springer-Verlag, New York. pp. 11-23.

Omata, T., Iwamoto, N., Kimura, T., Tanaka, A., and Fukui, S. 1981. Stereoselective hydrolysis of dl-menthyl succinate by gel-entrapped Rhodotorula minuta var. texensis cells in organic solvent. Eur. J. Appl. Microbiol. Biotechnol. 11: 199-204.

Osborn M.J. and Munson R. 1971. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Meth. Enzymol. 31: 642-653.

Penefsky, H.S. and Tzagoloff, A. 1971. Extraction of water-soluble enzymes and proteins from membranes. Meth. Enzymol. 22: 204-219.

Rama Devi, J., and Bhattacharyya, P.K. 1977a. Microbiological transformations of terpenes: Part XXIII. Fermentation of geraniol, nerol, and limonene by a soil pseudomonad, Pseudomonas incognita (linalool strain). Indian J. Biochem. Biophys. 14: 288-291.

Rama Devi, J., and Bhattacharyya, P.K. 1977b. Microbiological transformations of terpenes: Part XXIV. Pathways of degradation of geraniol, nerol, and limonene Psedomonas incognita (linalool strain). Indian J. Biochem. Biophys. 14:359-363.

Ray, A. 1971. Solvophobic interactions and micelle formation in structure forming nonaqueous solvents. Nature 231: 313-315.

Schwinghamer, E.A. 1980. A method for improved lysis of some gram-negative bacteria. FEMS Microbiol. Lett. 7: 157-162.

Scopes, R.K. 1987. "Protein Purification: Principles and Practice," 2nd ed. Springer-Verlag, New York.

Segel, I.H. 1976. "Biochemical Calculations," 2nd ed. John Wiley & Sons, Inc., New York.

Seidell, A. 1928. "Solubilities of Inorganic and Organic Compounds," 2nd ed. D. Van Nostrand Company, Inc., New York. p. 1466.

Seubert, W. 1960. Degradation of isoprenoid compounds by microorganisms. I. Isolation and characterization of an isoprenoid degrading bacterium, Pseudomonas citronellolis. J. Bacteriol. 79: 426-434.

Shaw, P.E. 1979. Review of quantitative analyses of citrus essential oils. J. Agric. Food Chem. 27(2): 246-257.

Simonsen, J.L. 1947a. Limonene, In "The Terpenes," Vol. 1, 2nd ed. Cambridge at the University Press, London. pp. 143-164.

Simonsen, J.L. 1947b. α-Terpineol. In "The Terpenes," Vol. 1, 2nd ed. Cambridge at the University Press, London. pp. 256-266.

Takagi, K., Mikami, Y., Minata, Y., Yajima, I., and Hayashi, K. 1969. Bacterial oxidation of limonene to carvone. Jap. Patent. 7,238,998.

Tzagoloff, A. and Penefsky, H.S. 1971. Extraction of lipoprotein complexes from membranes. Meth. Enzymol. 22: 219-230.

Vesterberg, O. 1971. Isoelectric focusing of proteins. Meth. Enzymol. 22: 389-412.

Walsh, C. 1979. "Enzymatic Reaction Mechanisms." W.H. Freeman and Co., San Fransico.

Wasserman, B.R. 1990. Evolution of enzyme technology: Progress and Prospects. Food Technol. 44(4): 118-122.

Welsh, F.W., Murray, W.D. and Williams, R.E. 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. Crit. Rev. Biotechnol. 9: 105-169.

West. S. 1987. Flavoring with enzymes. Food. November, pp. 39-43.

Whitaker, J.R. 1972. "Principles of Enzymology for the Food Sciences." Marcel Dekker, Inc., New York.

White, A., Handler, P., and Smith, E.L. 1964. " Principles of Biochemistry," 3rd ed. McGraw-Hill, Inc., New York.

Woodward, J. 1985. Immobilised enzymes: adsorption and covalent coupling. In "Immobilised Cells and Enzymes." Woodward, J. (Ed.). IRL Press, Washington D.C. pp. 3-17.

Yokozeki, K. Yamanaka, S., Takinami, K., Hirose, Y., Tanaka, A. and Sonomoto, K. 1982. Application of immobilized lipase to regio-specific interesterification of triglyceride in organic solvent. Eur. J. Appl. Microbiol. Biotechnol. 14: 1-5.

Zaks A. and Klibanov, A.M. 1988. Enzymatic catalysis in nonaqueous solvents. J. Biol. Chem. 263: 3194-3201.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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